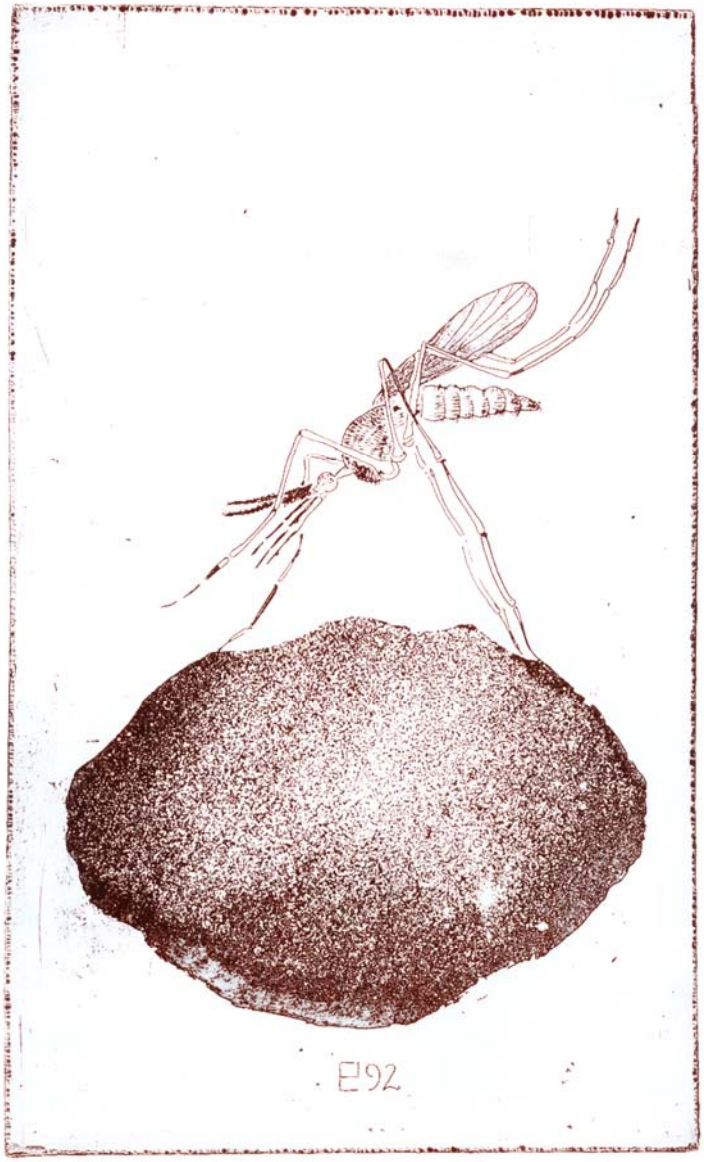
METHODS IN MALARIA RESEARCH



Sixth Edition

edited by

Kirsten Moll, Akira Kaneko, Arthur Scherf and Mats Wahlgren

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METHODS IN MALARIA RESEARCH 6th edition

Welcome to this new edition of **Methods in Malaria Research** which contains protocols provided by 122 scientists from the global malaria community. The manual is considered a "working document" that, with the help of our readers and users, will continuously grow and evolve as new and improved methods are developed. We, and the contributors hope that the manual will help and assist researchers at all stages of their careers in carrying out frontline research. We express our deep gratitude to all authors who have contributed to **Methods in Malaria Research** without whose efforts this new edition would not have been possible.

The manual was produced with funding from EVIMalaR a European Commission Network of Excellence Funded Project, Glasgow, U.K., MR4/BEI Resources at ATCC, Manassas, USA and Karolinska Institutet, Stockholm, Sweden. The book can be found online at www.evimalar.org, www.mr4.org, www.beiresources.org, and www.ki.se. Other valuable sources for protocols in malaria research are: Malaria Parasites and Other Haemosporidia. P.C.C. Garnham. Blackwell, Oxford, England, 1966; Malaria Methods and Protocols. Series: Methods in Molecular Medicine, vol. 72. D. L. Doolan (Ed.) 2002, Humana Press; Malaria Methods and Protocols. Methods in Molecular Biology, Vol. 923. R. Ménard (Ed.) 2nd ed. 2013.

If you have a method you think would be suitable for the next edition or you find some mistakes in the present edition, contact: Kirsten Moll, MTC, Karolinska Institutet, Nobels väg 16, Box 280, SE171 77 Stockholm, Sweden. e-mail: kirsten.moll@ki.se. For other comments or questions regarding the protocols, please contact the authors directly.

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PARASITES

I. Culturing of erythrocytic asexual stages of *Plasmodium falciparum* and *Plasmodium vivax*

I:A. The candle-jar technique of Trager–Jensen

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Equipment

incubator (37 °C)
glass desiccator (e.g., candle jar)
cell culture flask
candles
centrifuge
sterile pipettes
sterile tubes
glass slides and coverslips
microscope, fluorescence or light

Materials and reagents

purified erythrocytes (or human blood type O^+ in CPD-adenine (Terumo) or S.A.G.M. ("Sagman" solution or EDTA)
MCM (see below)
Tris (Sigma)
Albumax II (Gibco)
RPMI 1640 (Gibco)
gentamicin
1 M HEPES (Gibco)
Hanks' balanced salt solution (Gibco)
acridine orange (10 μg/mL) or Giemsa 5%

optional:
human serum
glucose
hypoxanthine (Sigma)
Tris-buffered Hanks' (TH)

Preparations

Prepare malaria culture medium (MCM) and Tris-buffered Hanks' (TH) for washing cells.

Albumax complete medium:

10.43 g RPMI 1640 powder (Gibco)
25 mL 1 M HEPES solution or 6 g HEPES (Gibco)
2 g $NaHCO_3$
0.5 mL gentamicin (from 50 mg/mL stock)
5 g Albumax II
Add distilled water to 1 liter. Filter-sterilize.
Use within 10 days, store at −20 °C.

Comment: For growing parasites from patient blood, use 10 g of Albumax for 1 liter of complete MCM. The vast majority of cultures will survive at least 2 weeks. It is also important to avoid serum in the culture for preparation of crude parasite antigen (see SEROLOGY, section III:B). Not all strains can be adopted to Albumax II medium.

Alternative MCM:

10.43 g RPMI 1640 powder (Gibco)
25 mL 1M HEPES or 6 g HEPES
2 g $NaHCO_3$
0.5 mL gentamicin (from 50 mg/mL stock)
Add distilled water to 1 liter. Filter-sterilize and store at −20 °C in 45-mL aliquots.

For complete MCM (cMCM), add 5 mL of human blood type AB^+ serum (inactivated at 56 °C for 60 min; then stored at −20 °C) to 45 mL of medium. Complete MCM can be used for up to one week if stored at 4 °C.

MCM can also be made from commercial liquid RPMI with sodium bicarbonate and HEPES buffer (Gibco). Just add 5 mL of 100× L-glutamine (Gibco) and 0.25 mL gentamicin (Gibco) to a 500 mL bottle of the RPMI.

TH (0.15 M Tris-buffered Hanks') (pH 7.2):

2.11 g Tris–HCl
0.2 g Tris-base
7.88 g NaCl
Dissolve in distilled water and bring volume to 1 liter.
Mix 1 volume of Tris buffer with 1 volume of Hanks'.

In vitro cultures in tissue-culture flasks

- Wash the erythrocytes 3 times in TH or RPMI 1640 to remove CPD, serum, and leukocytes if present. Dilute to 5% hematocrit with cMCM in small flasks of 25 cm^2 (0.2 mL of packed cells to 4 mL of cMCM) or in 75-cm^2 flasks (1.0 mL to 20 mL).
- Add parasites to an appropriate parasitemia (see below).
- Put the flask in a candle jar and loosen the screw cap. Produce low oxygen by burnt out candle and place the jar at 37 °C.
- Replace the MCM every day (not necessary the day after subcultivation).
- Subculture the cultures 2 times/week.

Subcultivation

- Stain a drop of the culture with acridine orange (10 µg /mL) on a glass slide and put on a coverslip or by Giemsa staining of a thin smear (see PARASITES, section III:A or B).
- Count the parasitemia (i.e., the percentage of infected cells, see PARASITES, section III:C).
- Prepare freshly washed O^+ blood in cMCM (5% hematocrit) and add it to the culture to obtain a parasitemia of not more than 1%, preferably 0.1 to 0.5% if two cycles until next subculturing, 0.5 to 1% if one cycle. Parasitemia should never exceed 15%.

References

Cranmer SL, Magowan C, Liang J, Coppel RL, Cooke BM. 1997. An alternative to serum for cultivation of *Plasmodium falciparum* in vitro. Trans R Trop Med Hyg 91(3):363-365.

Trager W, Jensen JB. 1976. Human malaria parasites in continuous culture. Science 193(4254):673-675.

I:B. Establishment of long-term *in vitro* cultures of *Plasmodium falciparum* from patient blood

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Careful adherence to the procedures described below should allow a very high success rate with immediate culture (provided parasites are not already damaged by antimalarial drugs), and even when using cryopreserved parasite stabilates, a success rate of >70% should be within reach.

Equipment

centrifuge
incubator

Materials and reagents

culture medium:
- 500 mL RPMI 1640 (Gibco)
- 25 mg gentamycin (Gibco)
- 91.6 mg L-glutamine (Sigma)
- 2.5 g Albumax II (Invitrogen) (See comments below.)
- 10 mL normal human serum
- 10 mg hypoxanthine (Sigma) (See comments below.)

washing medium:
- 500 mL RPMI 1640
- 25 mg gentamycin (Gibco)

freezing solution:
- sterile, distilled water
- 3.0% sorbitol (Sigma)
- 28.0% glycerol (Sigma)
- 0.65% NaCl

thawing solution:
- sterile, distilled water
- 3.5% NaCl

Giemsa stain:
- 10% Giemsa in phosphate buffer

gas mixture:
- 2% O_2, 5.5% CO_2, 92.5% N_2 (Special gas mixtures such as this one can be bought from suppliers of compressed gases.)

red blood cells (RBC) for subcultivation:
- Blood type O Rh^- or O Rh^+ RBC in CPD buffer, washed 3 times in washing medium to remove the Buffy coat (See comments below.)

Step-by-step manipulations

- Collect heparin or CPD anticoagulated venous blood from patient. Do not use EDTA blood, which does not support parasite survival. The collected blood can be used for cultivation immediately (“straight out of the arm”) or after cryopreservation.

Preparation for immediate cultivation (“straight out of the arm”) (optional)

- Wash the anticoagulated blood sample 3 times in prewarmed (37 °C) washing medium (centrifuged for 8 min at 600 × *g*). Remove buffy coat after each centrifugation.
- Continue as described under *Cultivation of fresh patient isolates* below.

Cryopreservation (optional—Do only if immediate culture is not possible or desirable.)

- Spin the anticoagulated blood sample (centrifuged for 8 min at 600 × *g*) and remove plasma.
- Mix equal amounts of ring-stage parasitized packed RBC and freezing solution (see comments below).
- Distribute the mixture immediately into 1.8-mL screw-cap tubes and snap-freeze in liquid nitrogen.
- Store the tubes in liquid nitrogen until use.
- On day of use, thaw the RBC suspension in a 37 °C-water bath.
- Spin the tube (8 min at 600 × *g*) immediately after thawing (see comments below).
- Discard the supernatant, add an equal amount of thawing solution, mix well, and incubate the cells for 1 min (see comments below).
- Fill the tube with prewarmed (37 °C) washing medium and repeat centrifugation as above.
- Wash the RBC 2 times in prewarmed (37 °C) washing medium (centrifuge for 8 min at 600 × *g*).
- Continue as described under *Cultivation of fresh patient isolates* below.

Cultivation of fresh patient isolates

- Suspend RBC at ~4% hematocrit in prewarmed (37 °C) culture medium.
- Add 5 mL of the RBC suspension to a 50-mL cell culture flask (T flask) or 25 mL to a 250-mL cell culture flask.
- Flush 50-mL flasks for ~30 s with gas mixture (250-mL flasks for ~90 s) at 1.5- to 2-bar pressure (see figure and comments below).
- Incubate the flasks at 37 °C for 24 h.
- Remove the culture flask gently from the incubator and remove spent supernatant.
- Remove a tiny amount of RBC with a sterile Pasteur pipette and use these RBC to make a Giemsa-stained thin smear.
- Add new (prewarmed) culture medium to the flask, gas it as above, and return it to incubation at 37 °C.
- Subcultivate the parasites when necessary (see comments below).

Comments

- The Albumax II and hypoxanthine we use cannot be considered sterile. We thus make a stock solution of 100 g of Albumax II and 400 mg of hypoxanthine in 2 liters of RPMI 1640 and filter the solution through 0.8-μm and 0.2-μm filters. We then use 50 mL of this stock solution per 500-mL bottle of culture medium.
- It is important to keep all media preheated to 37 °C and to minimize handling time outside the 37 °C incubator. (No coffee breaks while handling cultures!)
- When gassing the culture flasks, fit the gas hose with a 0.2-μm filter unit, and use a sterile needle (preferably blunt to avoid accidents) (see the figure).
- If initial patient parasitemia is high (>0.4%), change the medium the next day, otherwise leave it for 48 to 72 h before the first change of culture medium.
- Subcultivate patient isolates to keep parasitemia below 1 to 2% by adding fresh uninfected RBC. Although many laboratory-adapted parasite lines tolerate 5% parasitemia or more, this is not the case for most patient isolates.
- Do not leave parasites in freezing or thawing solution longer than absolutely necessary, as these solutions are harmful to the parasites.
- Washed uninfected RBC for subculture should be kept in the refrigerator for 24 h before first usage (to discourage any remaining leukocytes), and can be kept in the refrigerator for up to 14 days.

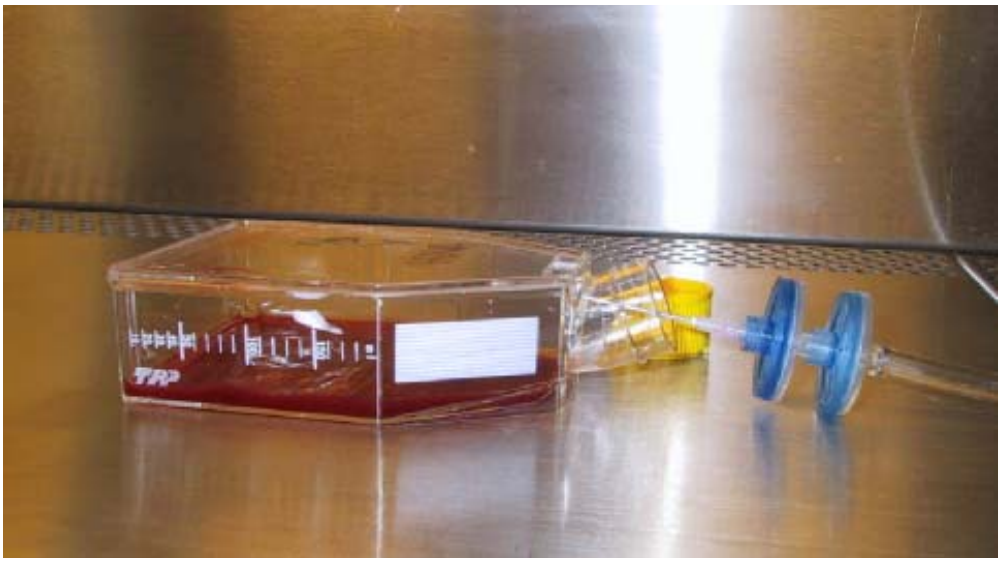
References
Cranmer SL Magowan C, Liang J, Coppel RL, Cooke BM. 1997. An alternative to serum for cultivation of *Plasmodium falciparum* in vitro. Trans R Soc Trop Med Hyg 91(3):363-365.

Jensen JB, Trager W. 1977. *Plasmodium falciparum* in culture: use of outdated erthrocytes and description of the candle jar method. J Parasitol 63(5):883-886.

Nielsen MA, Staalsoe T, Kurtzhals JA, Goka BQ, Dodoo D, Alifrangis M, Theander TG, Akanmori BD, Hviid L. 2002. *Plasmodium falciparum* variant surface antigen expression varies between isolates causing severe and non-severe malaria and is modified by acquired immunity. J Immunol 168(7):3444-3450.

Trager W, Jensen JB. 1976. Human malaria parasites in continuous culture. Science 193(4254):673-675.

Figure



I:C. Short-term cultivation of *Plasmodium falciparum* isolates from patient blood

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We give here a protocol for growing *P. falciparum* from patient blood. The vast majority of cultures will survive only the first cycle (i.e., from the ring stage to trophozoites/schizonts) and will not be able to reinvade new RBCs. In our hands, about 90% of patient isolates grew for at least one cycle. For establishment of long-term cultivation, see PARASITES section I:B, E.

Equipment

37 °C shaker incubator
centrifuge for 500-3000 × *g*
candle-jar or gas (90% N_2, 5% CO_2, 5% O_2)

Materials and reagents

venous blood from malaria patient
heparinized or EDTA tubes
Polymorphprep (Axis-Shield)
RPMI 1640 (optimal HEPES-buffered)
O^+ RBCs (or autologous blood group)
MCM with AB^+ serum
culture flasks or plates

Procedure

- Collect 2 to 5 mL of venous blood into heparinized/EDTA tubes and keep at 4 °C. Process the sample within 1 hour.
- Carefully layer the blood over 5 mL of Polymorphprep and centrifuge at 500 x *g* for 15 min.
- Collect the cell layer of interest. Erythrocytes are in the bottom of the tube.
- Add 10 mL of sterile RPMI 1640 (37 °C) to the cells, resuspend, and centrifuge at 500 x *g* for 5 min.
- Aspirate the supernatant and repeat the wash twice.
- Continue as described under *Cultivation of fresh patient isolates* in PARASITES, section I:B

If you wish to keep the monocytes, transfer the cell layer containing monocytes into a sterile 15-mL tube (see the manufacturer's protocol for Polymorphprep). Wash once with RPMI 1640 by centrifugation at 3,000 to 5,000 x *g* for 2 min. Remove the supernatant and add 1 mL of 10% DMSO in fetal calf serum. Freeze the cells immediately at –70 °C and then transfer to liquid nitrogen.

Reference:

Haeggström M, Kironde F, Berzins K, Chen Q, Wahlgren M, Fernandez V. 2004. Common trafficking pathway for variant antigens destined for the surface of the *Plasmodium falciparum*-infected erythrocyte. Mol Biochem Parasitol 133(1):1-14.

I:D. Growing *Plasmodium falciparum* cultures at high parasitemia

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By using increasing volumes of culture medium, one can grow *P. falciparum* to higher parasitemia (above 10%). This protocol may be especially suited for maintaining high parasitemias of wild isolates, allowing maturation of a maximum number of ring stage parasites to trophozoites.

Procedure

- Set up cultures as described in PARASITES, section I:A, but use the table below as a guide for medium volumes.
- Increase the parasitemia gradually using the indicated volumes until a desired parasitemia is reached, then maintain the culture in the appropriate volume.
  For example, for a 200-μL packed cell culture, use 4 times the volume of medium in the table for any given parasitemia.

% Parasitemia	Volume of medium (mL)/50 μL of packed culture
10	2.5
12	3
14	3.5
16	4
18	4.5
20	5
24	6
28	7
32	8
36	9
40	10
44	11
48	12
52	13
56	14
60	15
64	16
68	17
72	18
76	19
80	20
84	21
88	22
92	23
96	24
100	25

Reference

KEMRI, CGMRC / Wellcome Trust Research Unit, Kilifi, Kenya

I:E. Establishing and growing *Plasmodium falciparum* patient isolates *in vitro* with preserved multiplication, invasion and rosetting phenotypes

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Propagation of *P. falciparum* at optimal conditions *in vitro* is of fundamental importance for genotypic and phenotypic studies of both established and fresh clinical isolates. The method described here gave an outgrowth rate of 100% when tested on 76 frozen patient isolates. These optimized *in vitro* growth conditions to establish cultures of *P. falciparum* isolates requires orbital shaking (50 rev/min), presence of human serum (10 %/12.5%) and a fixed gas composition (5% O2, 5% CO2, 90% N2). In addition to improved outgrowth, the adhesive phenotype (rosetting) of the parasite isolates grown according to this protocol was preserved better as compared to standard procedures.
The protocol follows the set-up for culture regarding medium/serum described in chapter I.A with modifications for the growing conditions of the cultures.

Equipment

centrifuge
incubator
orbital shaker
equipment for gassing of cultures (5% O2, 5% CO2, 90% N2)

Materials and reagents

see chapter I.A

Procedure

- thaw isolates according to standard procedures
- after thawing, transfer pRBC to 5 ml malaria culture medium
- carry out cultivation in tissue culture flasks of 25cm2 (Falcon, nr. 353082)
- use 12.5% non-immune human AB+ serum and fresh O+ RBCs for cultivation
- grow isolates in the presence of a gas mixture **(5% O2 and 5% CO2 in N2)**
- grow isolates in suspension **on an orbital shaker (50 rpm)**

References

Ulf Ribacke, Kirsten Moll, Letusa Albrecht, Hodan Ahmed Ismail, Johan Normark, Emilie Flaberg, Laszlo Szekely, Kjell Hultenby, Kristina E.M. Persson, Thomas G Egwang, Mats Wahlgren (2013) Improved *in vitro* culture of *Plasmodium falciparum* permits establishment of clinical isolates with preserved multiplication, invasion and rosetting phenotypes. Plos One, *under revision*

I:F. Arresting *Plasmodium falciparum* growth at the trophozoite stage with aphidicolin

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This is a method to get fresh/frozen isolates or in vitro cultures to arrest in late trophozoite stages by inhibiting DNA synthesis.

Materials and reagents

malaria culture (only young stages! see PARASITES, section I:A) or a vial of frozen malaria parasites
aphidicolin (Sigma-Aldrich)
DMSO

Preparations

- Prepare a 1.5 mg/mL stock-solution of aphidicolin in DMSO.
- Dilute stock-solution 1:1000 into your malaria culture (4 μL in a 4-mL flask).
- Let the culture continue to grow until the parasites reach trophozoite stage (around 24 h after adding the aphidicolin). See PARASITES, section I:A.

Analyse or use your culture within 12 h after trophozoite stages are reached. Even though the parasites may look fine for some more hours, it is NOT recommended to use them after a longer time in aphidicolin.

Note: If you add the aphidicolin to a culture of later stages, the cells will not arrest.

References

Bull P, KEMRI, CGMRC, Kilifi, Kenya (personal communication)

Inselburg J, Banyal HS. 1984. *Plasmodium falciparum:* synchronization of asexual development with aphidicolin, a DNA synthesis inhibitor. Exp Parasitol 57(1):48-54.

I:G. *In vitro* growth of PfEMP1-monovariant parasite culture

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Equipment

Vario-MACS magnet (Miltenyi Biotec)
MACS LS-column (Miltenyi Biotec)
rotator
centrifuge

Materials and reagents

Culture flask, 25 cm2
Parasite culture of 5 to 10% parasitemia
Malaria culture medium (MCM; see: Parasites, I.A.)
RPMI 1640
PBS (pH 7.2) with 2% Fetal Calf Serum (FCS)
Anti-mouse IgG microbeads (Miltenyi Biotec)
specific anti-PfEMP1 antibodies

Procedure

- Perform the experiment on culture with 5% hematocrit, 4 to 8% parasitemia at late stage, optimal results are obtained when rosetting rate is above 30%. Several consecutive rounds of enrichment can be employed if needed.
- Wash the culture twice (at 500x *g*) with 2% PBS/FCS
- Block for 30 minutes in 2% PBS/FCS at room temperature in rotation.
- Spin for 2 minutes at 500x *g* and resuspend packed PRBC in 200μl of mAb-solution in 2% PBS/FCS (final concentration 25μg/ml)
- Incubate for 1 hour at room temperature in rotation
- Wash twice (at 500x *g*) with 2% PBS/FCS
- Resuspend in 160μl of 2% PBS/FCS
- Add 40μl of anti-mouse beads (change the species if using other kind of mAbs)
- Incubate for 15 minutes at room temperature in rotation
- Add 1ml of 2% PBS/FCS and spin for 10 minutes at 300x *g*
- Wash twice (at 500x *g*) with 2% PBS/FCS
- Resuspend in 1ml 2% PBS/FCS
- Prepare the column: place the MACS LS-column in the Vario-MACS magnet stand
- Wash the column with 3ml of 2% PBS/FCS
- Apply the parasite suspension
- Wash the column three times with 3ml of 2% PBS/FCS
- Add 5ml of 2% PBS/FCS to the column, take it out from the MACS and eject it using the plunger
- Spin at 500x *g* for 2 minutes
- Wash twice (at 500x *g*) with RPMI
- Resuspend the pellet in 3.8ml MCM, add 150μl of fresh RBC and put back into culture

I:H. Culturing erythrocytic stages of *Plasmodium vivax*

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Equipment

CF11-column (Cellulose powder CF11, Whatman)
syringe
centrifuge
orbital shaker (Leigh Labs)
fluorescence or light microscope

Materials and reagents

PBS
McCoy´s 5A medium (Sigma–Aldrich)
reticulocyte-enriched red blood cells (may be obtained from cord blood or from hematochromatosis patients)
human AB serum
ethidium bromide (Sigma)
CAUTION: This chemical is toxic and mutagenic. Wear protective clothing and use only in a chemical fume hood.
brilliant cresyl blue (ALD, Sigma–Aldrich)
slides and coverslips

Procedure

- Collect 5 mL of heparinised blood from *P. vivax*-infected patients. Prepare thin and thick blood films for examination of parasite stages and parasitemia.
- Dilute the blood with 5 mL of PBS.
- Pack a sterile CF11-column having the same volume as the diluted blood (10 mL) in a syringe and equilibrate it with PBS before use. Add the diluted blood to the column and elute the red blood cells (RBC, reticulocyte enriched) with PBS. The leukocytes will stick to the CF11 cellulose.
- Collect the RBC passing through the column (reticulocyte enriched), centrifuge at 2,000 rpm, and wash once in McCoy's 5A medium at room temperature.
- Resuspend the RBC to 5% hematocrit in McCoy's 5A medium containing 25% human AB serum and maintain the culture in a candle jar or use a gas mixture containing 5% CO_2, 5% O_2, 90% N_2.
- In order to enhance growth of parasites, shake the culture gently and continuously at 75 rpm in the orbital shaker.
- Check the blood smears daily to confirm maturation of the parasite and add reticulocyte-enriched RBC when required.
- Enumerate the infected RBC using one of the following techniques:
  - Mix 1 drop of ethidium bromide (10 μg/mL) with one drop of the culture, mount the mixture on a glass slide with a coverslip, examine it under a UV-light microscope, and count the number of the parasitised RBC from 10,000 RBC.
  - Mix 1 volume of 1% brilliant cresyl blue in 5 volumes of the culture. Mount and examine as described above.

References

Chotivanich K, Silamut K, Udomsangpetch R, Stepniewska KA, Pukrittayakamee S, Looareesuwan S, White NJ. 2001. Ex-vivo short-term culture and developmental assessment of *Plasmodium vivax*. Trans R Soc Trop Med Hyg 95(6):677-680.

Udomsangpetch R, Socci R, Williams J, Sattabongkot J. Modified techniques to establish a continuous culture of *Plasmodium vivax* [abstract]. 2002. In: American Society of Tropical Medicine and Hygiene 51st Annual Meeting program; 2002 Nov 10-14; Denver, CO.

I:I. *In vitro* continuous culture of *P. vivax* by erythroid cells derived from HSCs

by ***Tasanee Panichakul[1] and Rachanee Udomsangpetch[2]***
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The protocol of in vitro continuous culture of *P. vivax* by erythroid cells derived from human cord blood HSCs was established by Panichakul, et al, 2007. This protocol includes 2 parts.

Part 1. Production of erythroid cells from HSCs, including
- 1.1Isolation of HSCs
- 1.2Culture of erythroid cells derived from HSCs
- 1.3Determination of erythroid cell markers
- 1.4Determination of cellular morphology of RBCs and reticulocytes
- 1.5Separation of erythroid cell fraction
- 1.6Cryopreservation and thawing method of HSCs/erythroid progenitor cells

Part 2. Cultivation of *P. vivax* by erythroid cells, including
2.1 Separation of blood stages of *P. vivax*
2.2 Co-culture of *P. vivax* with erythroid cells
2.3 Continuous culture of *P. vivax*
2.4 Detection of parasites in cultures

Materials and reagents

see appendix

Part 1. Production of erythroid cells from HSCs

1.1 Isolation of hematopoietic stem cells (HSCs) from human cord blood

1.1.1 Separation of mononuclear cells
- Human umbilical cord blood samples from normal full-term delivery is layer on LymphoPrep™ with ratio 1:1 by volume and then spin 1,200 g by centrifugation for 20 min at 20^{o}C without applying a brake.
- The mononuclear cell fraction is collected and wash 2 times with PBS/2mM EDTA buffer.
- The numbers of mononuclear cells are counted with trypan blue exclusion test.

1.1.2 Isolation of hematopoietic stem cells
- MNC, 1-2 x 10^8 cells in 1 ml of cold PBS/ 2 mM EDTA/ 0.5%BSA buffer are reacted with 100 µl of each of anti-CD133 antibody coated with microbead and anti-FcR antibody, at 4^{o}C, for 30 min.
- After washing with cold PBS/ 2 mM EDTA/ 0.5%BSA buffer, cells are applied onto wetting MS column.
- Following by adding cold PBS/ 2 mM EDTA/ 0.5%BSA buffer onto the column for removing non-binding cells.
- step3 is repeated for 4 -5 times
- $CD133^+$ cells are then flushed from the column. The isolated HSCs/$CD\ 133^+$ cells were cultured.

1.2 Culture of erythroid cells derived from HSCs

- HSCs/CD133$^+$ cells, 1-2 x 10^5 cell/ml, in 6-well tissue culture plate are cultured in 4 ml of Complete StemlineII medium supplemented with stem cell factor (SCF), IL-3, hydrocortisone, transferring, humulin® N, ferrous sulfate, monothioglycerol and erythropoietin (EPO).
- The first step of culture is the cell expansion for 8 days. On day 4, cells are diluted in 4 volumes of fresh complete Stemline II medium as described above, distributed into 4 wells, and continuously cultured for other 4 days.
- In the second step, cells (1 x 10^6 cell/ml) are transferred into 25 cm^2 tissue culture flask and cultured for 4 days in StemlineII medium with transferrin, humulin® N, ferrous sulfate, monothioglycerol and EPO, without SCF, IL-3 and hydrocortisone.
- In the third step (8 days), cells (1- 2 x 10^6) cell/ml are cultured in StemlineII medium supplemented with 10% human AB serum, transferrin, humulin® N, ferrous sulfate, monothioglycerol and without EPO. The culture medium is changed every 4 days in this last step.
- All cultures are incubated in the CO_2 incubator at 37^{o}C with 5% CO_2.

1.3 Determination of erythroid cell markers

- Markers of erythroid cells, CD71, CD45 and glycophorin A are by flow cytometry.
- Cells, 5-10 x 10^4 in 100 µl of medium are stained with 5 µl of each fluorescence-conjugated anti- CD 71, CD 45 and glycophorin A antibodies for 30 min, at 4^{o}C.
- Wash twice with PBS and then centrifuged at 1,000g for 5 min.
- Thereafter, the stained cells are fixed with 400 µl of 1% paraformaldehyde for acquisition and analysis by flow cytometry.
- The IgG isotype antibodies were used in parallel to adjust the baseline level.

1.4 Determination of cellular morphology of RBCs and reticulocytes

- Giemsa and Brilliant cresyl blue dyes are used for determining morphology of erythroid cells and reticulocytes, respectively.
- Cells, 5-10 x 10^4 in 100 µl of medium are spun onto a slide by cytospin, at 800 rpm for 10 min.
- Then, fixed with 95% ethanol for 5 min.
- Thereafter, stained for 15 min with Giemsa diluted with PBS (ratio 1:4 by volume), and washed with tap water.
- Stained slides were dried at room temperature.
- For staining reticulocytes, 5-10 x 10^4 cells in 100 µl of medium are mixed with 5 µl of 1% Brillant cresyl blue (see Appendix)
- Then cytospined at 800 rpm for 10 min.
- All stained cells were examined under light microscope.

1.5 Separation of erythroid cell/red blood cell fraction

- Erythroid cell/red blood cell fraction is separated by discontinuous gradients (30-60%) of Percoll solution. The preparation of discontinuous gradients (30-60%) of Percoll solution is shown in appendix.
- Preparing Percoll gradients, 2 ml of 60% Percoll are the first layer into a 15 ml-sterilized plastic tube.
- Then follow with 2 ml of each layer of 50, 40 and 30% of Percoll, respectively.
- The suspension erythroid cells are applied on the top layer into the tube of Percoll discontinuous gradients.

- After centrifuge at 1,200 g for 20 min at 20 ^{o}C without applying a brake. Each cell fraction is separately collected and washed twice with RPMI medium by centrifugation, at 1,000 g for 10 min.
- The cell surface markers (CD71, CD 45 and glycophorin A), cell morphology and reticulocytes in each cell fraction were analyzed by flow cytometry, and Giemsa and Brilliant cresyl blue staining, respectively.
- Separated erythroid cells were used to culture vivax parasites.

1.6 Cryopreservation and thawing method of HSCs/erythroid progenitor cells

- HSCs/erythroid progenitor cells, 10-20 x 10^6 cells were resuspended in 1 ml of freezing medium (Cell Blanker®, Nihon Zenuaku Kohgyo, Japan).
- Frozen cells were kept at -70^{o}C overnight and then transferred into liquid nitrogen tank.
- For thawing method, the frozen cells are thaw at 37^{o}C immediately and then transferred into 6-well plate.
- Thawed cells, 5-10 x 10^6 cell/well were cultured in 4 ml of complete stemline II medium (see Appendix) after 1 to 2 days, cells were expanded and then cultured as shown in the method of production of erythroid cells from HSCs as described above.

Part 2. Cultivation of *P. vivax* by erythroid cells

2.1 Separation of blood stages of *P. vivax*

- Ten milliliters of blood from patient are collected and mixed with 10 µl of heparin.
- Centrifuged to collect the pack cells and then wash with McCoy medium.
- These pack cells were diluted with McCoy (1:2 by volume) and then filtrated by Plasmodipur filter for removing white blood cells.
- The separated red cells, adjusted to 20 % Hct in McCoy are laid onto 60 % of Percoll (see Appendix) in ratio 2:1 by volume and then centrifuged at 1,200 g for 20 min at 20^{o}C without applying a brake.
- Separated asexual parasites are washed twice with Mc Coy medium and then used for cultivation of parasites.

2.2 Co-culture of *P. vivax* with erythroid cells

- Asexual stages of *P. vivax* (schizont > 50 %) are co-cultured with erythroid cells from 14 - 20 day-old of erythroid cell cultures.
- Ratios of parasites and erythroid cells in co-cultures are between 1: 5 - 1:20.
- The co-cultures consisted of 5 % of pack cells in 1 ml of McCoy medium supplemented with 25% human AB serum.
- All cultures are incubated at 37^{o}C with 5% CO_2. Parasite – infected cells are examined by Geimsa staining or immunofluorescence assay (IFA).

2.3 Continuous culture of *P. vivax*

- Co-cultures of vivax parasites and erythroid cells are maintained in McCoy medium with 25% human serum at 37^{o}C in 5% CO_2.
- One hundred micro-liters of cells from co-culture are harvested and 100 µl of fresh erythroid cells are then replaced.
- Parasite-infected cells are determined daily by Giemsa staining or IFA using anti-vivax parasite antibody. Paras

2.4 Detection of parasites in cultures

- Immunofluorescence assay (IFA) and Giemsa staining are used to detect parasite infected cells.

- Cells from *P.vivax*-erythroid cultures 5-10 x 10^4 in 100 µl of medium are spun onto a slide by cytospin, at 800 rpm for 10 min.
- Then, fixed with 95% ethanol for 5 min.
- Thereafter, stained for 15 min with Giemsa diluted with PBS (ratio 1:4 by volume), and washed with tap water.
- Stained slides were dried at room temperature.
- All stained cells were examined under light microscope.
- For IFA, 5-10 x 10^4 cells in 100 µl of medium are spun onto a slide by cytospin, at 800 rpm for 10 min.
- Then fixed with cold acetone for 10 min, then block with 2% BSA for 30 min.
- Anti-vivax parasite antibody is added for 30 min.
- Wash 3 times with cold PBS, then add 2nd antibody conjugated with fluorescence for 30 min.
- Wash 3 times, then mount slide by using anti-fed, then stained cells are determined under fluorescence microscope.

Reference

Panichakul T., Sattabongkot J., Chotivanich K., Sirichaisinthop J., Cui L., Udomsangpetch R. Production of erythropoietic cells in vitro for continuous culture of *Plasmodium vivax*. Int. J. Parasitol. 2007, 37: 1551-1557.

Appendix

Culture media and reagents

Complete StemlineII medium
- StemlineII medium (Sigma-Aldrich Corporation, Missouri, USA) 1ml
- 10 µg/ml stem cell factor (SCF) (PeproTech, Rocky Hill, NJ, USA) 10µl
- 1 µg/ml IL-3 (R&D Systems, Inc., MN, USA)5µl
- 10^{-4} M hydrocortisone(Sigma-Aldrich) 10 µl
- 100 mg/ml transferrin(Sigma-Aldrich)1µl
- 10 mg/ml Humulin® N (Lilly Pharma Fertigung UND Distribution, Giessen, Germany)10µl
- 0.18 mg/ml ferrous sulfate (Sigma-Aldrich) 1µl
- 160 mM monothioglycerol (Sigma-Aldrich) 1 µl
- 2000 IU/ml erythropoietin (Cilag AG International, Zug, Switzerland) 2µl

Stem cell factor (SCF) 10µg/ml
- SCF100µg
- 10 mM acetic acid 200µl
- StemlineII medium10ml

IL-3 1µg/ml
- IL-310µg
- FBS10µl
- StemlineII medium10ml

Transferrin100mg/ml
- Transferrin 100mg
- StemlineII medium1ml

Hydrocortisone 10^{-4} M
- Hydrocortisone1mg
- StemlineII medium 20.6ml

Ferrous sulfate 0.18mg/ml
- Ferrous sulfate1.8mg
- H_2O 0.1ml
- StemlineII medium10ml

Monothioglycerol 160mM
- Monothioglycerol 0.1ml
- StemlineII medium 7.12ml

Phosphate buffer saline 0.01 M, pH 7.4 (PBS)
- NaCl 8.5g
- $Na_2H_2PO_4$ 1.35g
- $NaH_2PO_4H_2O$ 3.45g
- H_2O 1000ml
- Autoclave

PBS/2 mM EDTA
- EDTA 0.744g
- PBS, pH 7.41000ml
- Autoclave

PBS/ 2 mM EDTA/ 0.5% bovine serum
PBS/ 2 mM EDTA100ml
Bovine serum 0.5 ml

McCoy's medium
RPMI 1640
RPMI 10.4g
HEPES 3.57g
$NaHCO_3$ 2g
D-glucose 1.8g
Gentamycin 10 mg/ml 1ml
H_2O1000ml

Percoll 60%
Percoll 5. ml
10x RPM I0.6ml
RPMI 4.0ml

Percoll 50%
Percoll 4.5ml
10x RPM I0.5ml
RPMI 5.0ml

Percoll 40%
Percoll 3.6ml
10x RPM 0.4ml
RPMI 6.0ml

Percoll 30%
Percol l2.7ml
10x RPMI 0.3ml
RPMI 7.0ml

1% Brillant Cresyl Blue
Brillant Cresyl Blue 1g
NaCHO 0.4g
NaCl 0.9g
H_2O 100ml

Anticoagulant solution (CPDA-1)
Sodium citrate dehydrate 2.63g
Citric acid monohydrate 327mg
Glucose anhydrous 2.9g
Sodium dihydrogen phosphate dehydrate 251 mg
Adenine 27.5 mg
Water 100 ml

McCoy 11.9g
HEPES 3.57g
$NaHCO_3$ 2g
Gentamycin 1x 10^6 mg/ml 1ml
H_2O 1000ml

II. Freezing and thawing of asexual *Plasmodium* spp.

We give several methods below for cryopreserving and thawing *Plasmodium* strains and wild isolates. In general, parasites frozen according to one method can be thawed using another method's thawing protocol. For convenience, we generally freeze parasite strains using the Stockholm Sorbitol Method, while thawing using the simpler NaCl Method or the 5% & 27% Sorbitol Method. It appears that the lengthier thawing protocol of the Stockholm Sorbitol Method gives lower initial parasitemia (killing of the later stages) and a more synchronous culture during the first two cycles.

When parasites are cultured in Albumax II MCM to avoid adhering IgG, no addition of serum is required.

II:A. Stockholm Sorbitol Method

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Equipment

tabletop centrifuge (Beckman)
liquid nitrogen tank
immufuge

Materials and reagents

fetal bovine serum (Gibco)
freezing medium:
28% glycerol
3% sorbitol
0.65% NaCl
distilled water
To make 250 mL: Mix 180 mL of 4.2% sorbitol in 0.9% NaCl with 70 mL of glycerol. Filter-sterilize; store frozen.

Freezing

- Transfer a 4-mL culture into a test tube.
- Pellet erythrocytes by centrifugation (immufuge on low for 1 min) and discard the supernatant; the pellet is approximately 0.2 mL.
- Add 0.3 mL of serum (of complementary blood group) (40% hematocrit). For Albumax II cultures (see PARASITES, section I:A), add 0.3 mL of fetal bovine serum (40% hematocrit).
- Add 0.5 mL of the freezing medium, drop-by-drop, while shaking the vial gently; the addition should take approximately 1 min.
- Transfer the medium into a sterile cryovial.
- Drop the vial gently into the liquid nitrogen tank.

Thawing

- Prepare the sorbitol solutions listed below, have MCM and heat-inactivated serum ready (see PARASITES, section I:A). For Albumax II MCM, see also the comment above.

- Take a malaria culture vial from the liquid nitrogen tank.
- Thaw it quickly, approximately 1 min in a 37 °C-water bath.
- Put the vial on ice and transfer the contents into chilled 50-mL centrifuge tubes and centrifuge gently to pellet the cells (250 × *g* for 5 min at 4 °C in a Beckman tabletop centrifuge).

- Remove the supernatant and add the following, drop by drop, while shaking the vial gently:
  1 mL 17.5% sorbitol in ice-cold PBS, then
  2 mL 10% sorbitol, and finally
  2 mL 7.5% sorbitol

- Centrifuge as before, remove the supernatant, then add:
  1 mL 10% sorbitol, then
  2 mL 7.5% sorbitol, and finally
  2 mL 5% sorbitol

- Centrifuge, then add:
  1 mL 7.5% sorbitol, then
  2 mL 5% sorbitol, and finally
  2 mL 2.5% sorbitol

- Centrifuge, then add:
  1 mL 5% sorbitol, then
  2 mL 2.5% sorbitol, then
  2 mL MCM with 15% serum (at room temperature)

- Centrifuge, then add:
  1 mL 2.5% sorbitol, then
  2 mL MCM with 15% serum
  Transfer to a 5-mL tube.

- Centrifuge in the immufuge.
  Add 4 mL of MCM containing 15% serum (see comment for Albumax II MCM above) and packed erythrocytes of a complimentary blood group (see also PARASITES, section I:A).

II:B. Freezing of patient isolates and strains with glycerolyte

by ***Hodan Ahmed Ismail***
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This protocol is useful for all primate malaria parasites tested *(Plasmodium falciparum, P. vivax, P. knowlesi, P. coatneyi, P. fragile,* and *P. cynomolgi).*

Equipment

centrifuge for 500-3000 × *g*

Cryoprotective solution

glycerolyte:

57% glycerol USP
16 g/liter of sodium lactate
300 mg/L of KCl USP
25 mM sodium phosphate (pH 6.8)
(This solution may be purchased from Baxter/Fenwal.)

Procedure

All steps are at room temperature unless otherwise stated.

- Take heparinized blood containing very young ring stages of malaria and centrifuge it for 10 min at 700 × *g* (approximately 3,000 rpm).
- Remove the supernatant and estimate volume of packed cells, V.
- SLOWLY add 0.33 × V of glycerolyte through gentle mixing. Let the tube stand for 5 min.
- Add dropwise 1.33 × V of glycerolyte, mixing gently.
- Label cryotubes with the parasite's code/name and date.
- Distribute 1 mL of the preparation per cryovial.
- Freeze at −80 °C for at least 18 h.
- Transfer vials to liquid nitrogen or -150 ^{0}C freezers for long-term storage.

II.C. Thawing of glycerolyte-frozen parasites with NaCl

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Equipment

centrifuge for 500-3000 × *g*
candle-jar or gas (90% N_2, 5% CO_2, 5% O_2)
37 °C shaker incubator

Materials and reagents

sterile 1.6% NaCl (Baxter/Fenwal, 4B7870)
sterile 12% NaCl (Baxter/Fenwal, 4B7874)
malaria culture medium (MCM, see PARASITES, section I:A)
15% A^+ serum (for clinical isolates use AB^+ serum)

Procedure

- Remove a vial from cold storage and thaw it up quickly at 37 °C for 1 to 2 min.
- Transfer blood to 50-mL centrifuge tubes with a sterile pipette. Measure blood volume, V.
- Add 0.1 × V of 12% NaCl slowly, dropwise, while shaking the tube gently.
- Let the tube stand for 5 min.
- Add 10 × V of 1.6% NaCl slowly, dropwise, swirling the tube.
- Centrifuge the tube at 500 × *g* at 20 °C for 5 min.
- Aspirate the supernatant and add 10 × V of MCM slowly, dropwise, while shaking the tube.
- Centrifuge the tube at 1,500 rpm at 20 °C for 5 min and aspirate the supernatant.
- Resuspend pelleted blood cells in MCM with 15% A^+ serum and transfer to a culture flask using the candle-jar method. NOTE if thawing up clinical isolates gas the culture flasks.
- For continued culturing, see PARASITES, section I.

II:D. Thawing of cryopreserved *Plasmodium falciparum* using sorbitol

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Equipment

water bath (37 °C)
table-top centrifuge (Beckman)

Materials and reagents

27% sorbitol in PBS, filter-sterilized, stored at 4 °C
5% sorbitol in PBS, filter-sterilized, stored at 4 °C
15% AB^+ serum

Procedure

- Remove a vial from liquid nitrogen and thaw it quickly in a 37 °C water bath; shake it lightly.
- Transfer thawed parasites into a round-bottomed 13-mL tube (max. 1 mL/vial).
- Slowly add 2 volumes of 27% sorbitol, the first volume over 8 min, the second over 5 min. Slowly stir the tube constantly on a vortex.
- Let it stand for 5 min.
- Add 2 volumes of 5% sorbitol over 10 min.
- Let it stand for 5 min.
- Centrifuge the tube at 400 × *g* (1,250 rpm on a table-top Beckman centrifuge) for 3 min; discard the supernatant.
- Add 2 volumes of 5% sorbitol over 8 min.
- Let it stand for 5 min.
- Centrifuge the tube at 400 × *g* for 3 min; discard the supernatant.
- Add 1 to 2 mL of MCM over 1 min. Centrifuge the tube at 400 × *g* and discard the supernatant. The wash may be repeated once.
- Resuspend the pellet in 15% AB^+ serum in MCM. Add washed, packed RBC's to 5% hematocrit.
- Change the medium the next day. Continue culturing as usual (see PARASITES, section I).

Reference

Procedures received from M. Hommel, modified from:
Diggs C, Joseph K, Flemmings B, Snodgrass R, Hines F. 1975. Protein synthesis in vitro by cryopreserved *Plasmodium falciparum*. Am J Trop Med Hyg 24(5):760-763.

III. Staining of parasite culture or patient blood and estimation of parasitemia

III:A. Acridine orange (AO) vital stain of cultures

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Equipment

UV-equipped microscope

Materials and reagents

Acridine orange (AO) (Fluka)

Stock solution (10x):

Dissolve 10 mg of AO in 10 mL of distilled water. Add sodium azide to a final concentration of 0.02%. Store in the dark at 4 °C.

Working solution:

Add 0.1 mL of stock solution to 9.9 mL of PBS (pH 7.2) or RPMI-1640. Store in the dark at 4 °C.

Alternate acridine orange hydrochloride solution (Fluka)

Dilute 1:5000 in PBS. Add sodium azide to a final concentration of 0.02%. Store in the dark at 4 °C.

Procedure

- Mix one drop of culture with a small drop of AO on a slide.
- Put on a coverslip.
- Analyse the slide immediately or keep it in the dark.
- Count 5 to 10 fields of vision to estimate the parasitemia (40× objective, 10× ocular).

III:B. Giemsa staining of thick or thin blood films

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Equipment light
microscope

Materials and reagents
Giemsa stain:
For best and most reproducible results, buy ready-made Giemsa stain from, for example, Sigma–Aldrich. It is quite difficult to prepare a batch that works as well as the commercial one.
Check every new batch of Giemsa stock solution before using it.
Prepare a new working solution for each staining session or at least every three hours.
Add one buffertablet for microscopy pH 7.2 (Merck) to one liter of tap water or prepare a phosfate buffer solution:

phosphate buffer, 6.7 mM (pH 7.1):
0.41 g KH2PO4
0.65 g Na2HPO4 □ H2OBring to 1 liter with water.
methanol (reagent grade for analysis, Merck)

Giemsa staining of thick blood films from **cultures**
- Leave the thick film to dry properly, preferably overnight. In case of hurry, leave it to dry at least 30 min or in an incubator at 37 °C for 15 min. Do not fix.
- Prepare a fresh 2% Giemsa solution in phosphate buffer
- Put the slide on a staining rack and pour the Giemsa solution on the slide. Leave it to stain for 30 min. (A stronger Giemsa solution will over-stain parasites from culture.)
- Rinse very carefully and gently in a beaker with fresh water. If tap water is used, let the stream runon the backside of the glass..
- Leave the slide in an upright position to dry.
- Observe with immersion oil and objective at 100·.

Giemsa staining of thick blood films from **patients**
- Leave the thick film to dry properly. In case of hurry, leave it to dry in an incubator at 37 °C for 30 min.
- Prepare a fresh 4 -5% Giemsa solution in phosphate buffer
- Put the slide on a staining rack and pour the Giemsa solution on the slide. Do not fix.
- Leave the slide to stain for 20 min.
- Rinse very carefully and gentlyin a beaker with fresh water. If tap water is used, let the stream runon the backside of the glass.Leave the slide in an upright position to dry.
- Observe with immersion oil and objective at 100·.

Giemsa staining of thin blood films from patients or cultures
- Air-dry the thin film.
- Fix the film in methanol for about 30 sek.
- Prepare a fresh 5 -10% Giemsa solution in phosphate buffer

- Put the slide in a staining jar or on a staining rack and pour the Giemsa solution on the slide.
- Leave it to stain for 20 min.
- Rinse it carefully and thoroughly under running tap water.
- Leave the slide in an upright position to dry.
- Observe the film with immersion oil and objective at 100·.

Reference

Malaria Working Party of The General Haematology Task Force of the British Committee for Standards in Haematology. 1997. The laboratory diagnosis of malaria. Clin Lab Haematol 19(3):165-170.

III:C. Estimation of the percentage of erythrocytes infected with *Plasmodium falciparum* in a thin blood film

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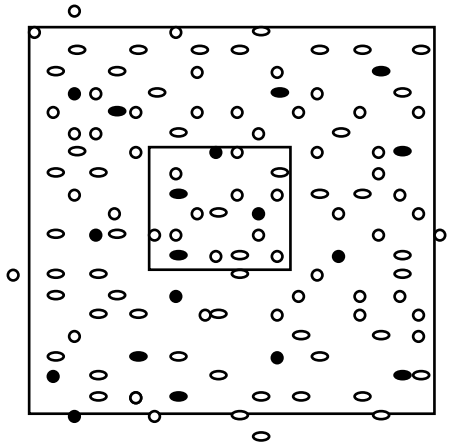
Equipment

Use a light microscope with a special ocular (i.e., a reticulocyte ocular) which has a large and a small square where the small square is 10% of the large one (see the figure below, although proportions are not maintained).

Procedure

- Use the 100· objective to view cells under oil immersion.
- Choose an area of a Giemsa-stained thin blood film where the erythrocytes are in a monolayer and evenly distributed.
- Count *all* erythrocytes in the small square plus two of the borders.
- Without moving the slide, also count the number of *infected erythrocytes* in the whole area of the big square plus two borders (i.e., including those in the area of the small square).
- Move the slide to "randomly adjacent" fields and continue counting as above (i.e., all erythrocytes of small fields and parasites of accompanying large fields). Continue counting fields until you reach the sum of 100 erythrocytes in the small fields. By extrapolating, the equivalent of 1000 erythrocytes have been examined (regarding parasites).
- Repeat the counting at least twice for a total examination of three different parts of the slide. Take the mean number of infected erythrocytes per 1000 erythrocytes and divide by 10 to get the percent infected erythrocytes.



Please note: If one erythrocyte contains **2 parasites** or more, it is still counted as **one** infected erythrocyte.
If no special ocular is available, count the number of erytrocytes and infected erytrocytes in the whole microscopic field before you move to next and continure the counting until at least 1000 erytrocytes are counted. Calculate the parasitemia as above.

For estimation of parasite density in thick film from patients see: Basic malaria microscopy WHO;1991

Reference

Brecker G, Schneiderman M. 1950. A time saving device for the counting of reticulocytes.Am J ClinPathol 20:1079-1083.

III:E. Estimating the rosetting rate of a parasite culture

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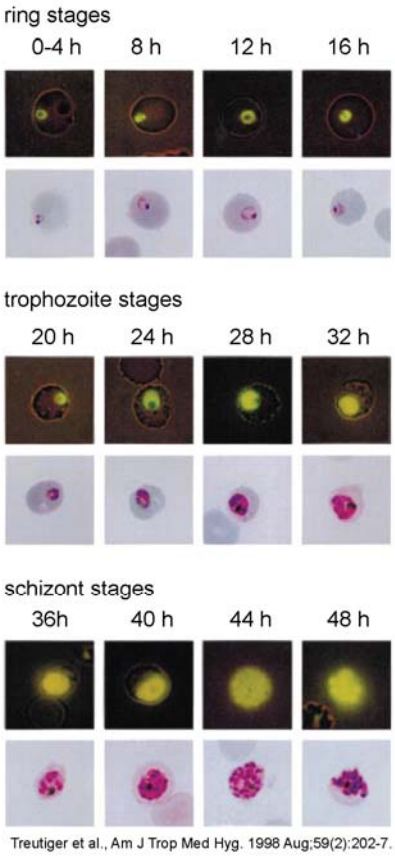
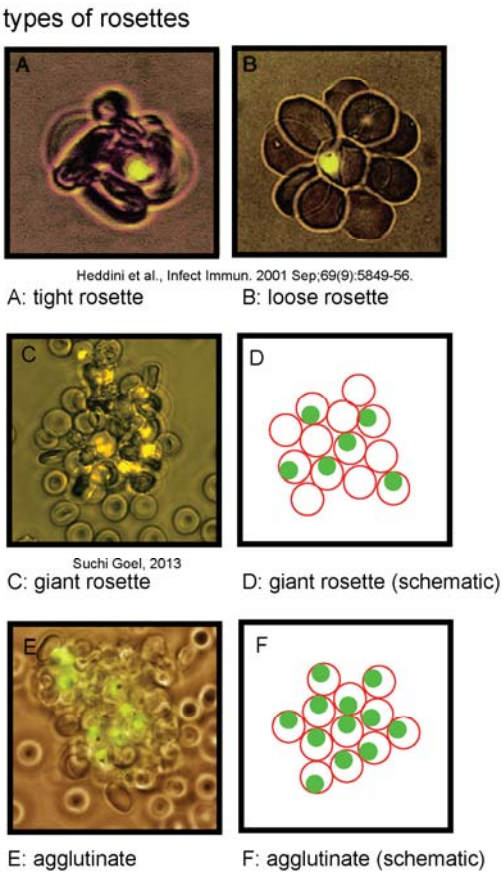
Equipment

Use a light microscope with preferably one of the oculars is equipped with a counting grid

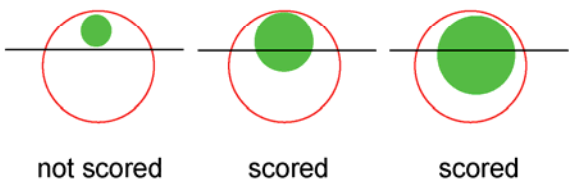
Procedure

- Stain the parasite culture with Acridine Orange (see chapter III.A)
- Use the 40x objective to view the parasite culture
- Count from upper left to lower right corner over the coverglass, field next to field (page 73)
- counting: count all pRBC, where the parasite fills 1/3 or more of the RBC (see picture below)





To score a pRBC in order to determine the rosetting rate of a culture, only pRBC that contain parasites filling the RBC to 1/3 of more are included:



III:E. Evaluation of parasitemia by lactate dehydrogenase assay

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Evaluation of parasitemia by lactate dehydrogenase assay is a high throughput method for screening of antimalarial factors.

Equipment

microplate reader
flat-bottomed 96-well plate

Materials and reagents

Malstat reagent (Flow Incorporated)
red blood cells
nitro blue tetrazolium (NBT) stock solution:
100 mg NBT
50 mL distilled water
Dissolve NBT in distilled water. Store in the dark at 4 ^{o}C.

phenazine ethosulphate (PES) stock solution:
5 mg of PES
50 mL distilled water
Dissolve PES in distilled water. Store in the dark at −20 ^{o}C.

Procedure

- Add 100 µL of Malstat reagent to 96-well microplate.
- Add 20 µL of infected or noninfected red blood cells (hematocrit is 1 to 2%).
- Incubate the plate at room temperature and shake gently several times during incubation to solubilize red blood cells.
- During incubation, mix equal volumes of NBT and PES in the dark and add 20 µL of the mixture to the wells.
- Keep the plate in the dark.
- After 30 to 60 min, check the color development (dark purple).
- Read the plate at 650 nm. Use uninfected red blood cells as reference.
- This assay can be used in 0 to 7% of parasitemia (non-synchronized parasite in 2% of hematocrit).

If you deal with a large number of samples, 20 µL of red blood cells in 96 wells can be stored in 96-well plates at −80 ^{o}C before the enzyme assay.

References

Gomez MS, Piper RC, Hunsaker LA, Royer RE, Deck LM, Makler MT, Vander Jagt DL. 1997. Substrate and cofactor specificity and selective inhibition of lactate dehydrogenase from the malarial parasite *P. falciparum*. Mol Biochem Parasitol 90(1):235-246.
Makler MT, Hinrichs DJ. 1993. Measurement of the lactate dehydrogenase activity of *Plasmodium falciparum* as an assessment of parasitemia. Am J Trop Med Hyg 48(2):205-210

Makler MT, Ries JM, Williams JA, Bancroft JE, Piper RC, Gibbins BL, Hinrichs DJ. 1993. Parasite lactate dehydrogenase as an assay for *Plasmodium falciparum* drug sensitivity. Am J Trop Med Hyg 48(6):739-741.

IV. Purification and synchronization of erythrocytic stages

IV:A. Enrichment of knob-infected erythrocytes using gelatine sedimentation

by ***Kirsten Moll***
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Equipment

immufuge blood centrifuge

Materials and reagents

mature parasite stages
gelatine (Sigma G2625)
RPMI 1640 with HEPES

Procedure

- Make 0.7% gelatine in RPMI 1640 with HEPES.
- Warm and stir the solution at 37 °C until the gelatine is dissolved.
- Filter-sterilize the solution and store it in the refrigerator. Warm it to 37 °C before use.
- Use mature parasite stages. (Ring forms, knobless late stages, and rosettes will sink to the bottom.)
- Wash the culture once using RPMI 1640.
- Resuspend the pellet, approximately 0.2 mL, in 2 mL of 0.7% gelatine solution.
- Incubate the pellet for 1 h at 37 °C.
- Carefully remove the supernatant, which contains the “knobby” parasites, without touching the pellet.
- Centrifuge the supernatant to pellet the parasitized RBC (PRBC) (1 min on high setting in the immufuge blood centrifuge).
- Wash the cells 2 or 3 times using RPMI 1640.
- Subculture the cells using fresh blood and media (see PARASITES, section I).

Reference

Goodyer ID, Johnson J, Eisenthal R, Hayes DJ. 1994. Purification of mature-stage *Plasmodium falciparum* by gelatine flotation. Ann Trop Med Parasitol 88(2):209-211.

IV:B. Sorbitol-synchronization of *Plasmodium falciparum*-infected erythrocytes

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Equipment

centrifuge

Materials and reagents

parasite culture of >5% parasitemia
sorbitol
malaria culture medium (MCM)

Procedure

- Take parasites when they are mostly at the ring stage. They must not be later than 10 to 12 h postinvasion when the sorbitol treatment is done.
- Spin down the parasite culture at 600 × *g* to a pellet (take 4 mL of a culture of >5% parasitemia).
- Add 4 mL of 5% sorbitol (in distilled water) and incubate for 10 min at room temperature. Shake 2 or 3 times.
- Centrifuge the culture at 600 × *g,* and wash it 3 times in malaria culture medium, and dilute it to 5% hematocrit.
- Count parasitemia and subculture as usual.
- Repeat the procedure after one cycle (approximately 48 h).
- To keep the parasites synchronized, the sorbitol treatment must be performed once a week.

Reference

Lambros C, Vanderberg JP. 1979. Synchronization of *Plasmodium falciparum* erythrocytic stages in culture. J Parasitol 65(3):418-420.

IV:C. Tight synchronisation protocol for *in vitro* cultures of *Plasmodium falciparum*

by ***Jonathan M. Mwangi, Georgina S. Humphreys, Abhinav Sinha and Lisa C. Ranford-Cartwright***
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Equipment

Centrifuge
Modular incubator (Billups-Rothenberg, USA)

Materials and reagents

Culture (5-10%) with high proportion of mature stage parasites
Plasmion (Fresenius, France)
Concanavalin A (Sigma)
35 mm tissue culture dishes

Procedure

- Collect 25 ml of parasite culture and centrifuge for 10 minutes at 232 x *g*.
- Discard the supernatant and resuspend the pellet in serum-free RPMI medium at a ratio of 3:1 medium: pellet.
- Add equal volume of Plasmion and incubate at 37^0C for 30 minutes to separate the solution into a pellet containing uninfected RBCs and ring stage parasites, and supernatant containing trophozoites and schizonts.
- Take out the supernatant into a fresh tube and centrifuge at 232 x *g* for 10 minutes to pellet the parasites and discard the supernatant. This pellet usually has about 60-70% parasitaemia made up of mostly trophozoites and schizonts.
- Resuspend the pellet in serum-free RPMI medium at a ratio of 10:1 pellet: RPMI for setting up monolayers.
- Coat sterile 35 mm tissue culture dishes with 1.5 ml of 10 µg/ml Concanavalin A and incubate at 37^0C for 1 hour.
- Gently remove excess Concanavalin A with three washes of serum-free RPMI medium.
- Pour 1.5 ml of the Plasmion treated culture onto the Concanavalin A-coated plate and incubate in a gassed modular incubator for 1 hr at 37^0C to form a schizonts-rich monolayer. Thoroughly wash off excess culture with 5 washes of serum-free RPMI medium.
- Make an overlay by pouring 1.5 ml of fresh uninfected RBCs (10 % haematocrit) onto the monolayer in the plate. Incubate the plate in the gassed modular incubator as above at 37^0C for 1 hour.
- Harvest the newly parasitized RBCs and put into a culture flask and continue as normal culture. This culture should contain merozoites that have invaded RBCs within 1 hour. A smear of the culture should be made at this time to confirm parasite invasion and to rule out any carryover of mature parasites from the monolayer. The same monolayer can be used to infect multiple overlays of fresh RBCs. In our experience, the second and third overlays have the best invasions with about 4-5% parasitaemia.

References

Ranford-Cartwright, L.C., Sinha, A., Humphreys, G.S. and Mwangi, J.M. (2010). New synchronisation method for *Plasmodium falciparum*. Malaria J. 9: 170

IV:D. Tight synchronization of *P. falciparum* asexual blood stages for transcriptional analysis

by ***Núria Rovira-Graells[1]***, ***Valerie M. Crowley[2]*** *and* ***Alfred Cortés[1,3]***
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[3]Catalan Institution for Research and Advanced Studies (ICREA), Barcelona, Spain

This protocol provides a method to tightly synchronize *P. falciparum* parasites for transcriptional analysis. Many *P. falciparum* genes show cyclical expression patterns along the asexual cycle, and even small differences in the stage of the parasites can result in large differences in the transcript levels observed. This can be an important source of false differences when comparing transcript levels between parasite lines (eg wild type vs transgenic parasite lines, etc.). Here we provide a protocol to obtain a population of parasites of a defined age window of 0-5 h, which can be then grown for transcriptional comparison at any stages of the intraerythrocytic cycle.

Equipment

Modular incubator chamber (Billups–Rothenberg)
Vario-MACS magnet (Miltenyi Biotec)
MACS CS-columns (Miltenyi Biotec)

Materials and reagents

Petri dishes
P. falciparum culture medium
Sorbitol
Percoll™ (GE Healthcare)
TRIzol®Reagent (Life Technologies)

Procedure

- Thaw parasites (day 0) and grow them under standard conditions. In our lab we culture parasites in Petri dishes at a 3%hematocrit in culture medium containing Albumax II and in a 5% CO_2, 3% O_2, 92% N_2 atmosphere in a modular incubator chamber.
- On day 3 (or when a parasitemia >2% is reached), determine the time at which substantial schizont bursting starts. Take smears at different times to estimate by microscopy the approximate time at which the rings/schizonts ratio is at least 1 (meaning that there are at least as many new rings as schizonts from the previous cycle).
- On day 4 (or when an adequate volume of culture at 5% rings is obtained), synchronize by sorbitol lysis using standard methods. We typically start the experiment with at least 20 ml of culture (0.6 ml of packed RBC) at a 5% parasitemia.
- The next day, take a smear at the time you expect a rings/schizonts ratio between 1 and 2 (typically between 24 and 36 h after sorbitol lysis, depending on the age of the parasites when sorbitol synchronization was performed. The information collected on the day before sorbitol can give you an idea of what time to start checking). If the ratio is higher than 1 (preferably higher than 2), you can proceed to the next step, otherwise place parasites back into culture and wait for

several hours until enough schizonts have burst. It is critically important to start the next step (purification of schizonts) when the rings/schizonts ratio is higher than 1 (preferably 2) to ensure that homogeneous bursting of schizonts occurs during the following 5 h.

- Purify schizonts by magnetic separation following standard protocols (for 3D7 and a few other parasite lines, 70% Percoll purification can be used instead of magnetic purification, but the yield of Percoll purification is low for the majority of other parasite lines). The purity should be 85-99% (85 to 99% of the erythrocytes should be schizont-infected).
- Mix purified schizont-infected RBCs with washed RBC (uninfected) to adjust the final parasitemia to 1-2.5% (check by microscopy, the amount of RBC added is typically about 75% of the RBCs in the starting culture).
- Place back into culture for exactly 5 h.
- Perform sorbitol lysis, and take a smear to estimate parasitemia. It is expected that the parasitemia of ring stage parasites will be similar to the starting parasitemia (before magnet or Percoll purification) and approximately double the parasitemia at the beginning of the 5 h incubation. This culture contains only rings 0-5 h post invasion.
- Distribute the culture in as many dishes as time points are needed for your transcriptional analysis. Since the amount of RNA in early stage parasites is lower than in late stage parasites, larger volumes of culture are required for early stages than for late stages.
- Let the parasites grow undisturbed for the number of hours that each time point requires. For example, culture for 25 h to have a population of parasites 25-30 h post invasion. Collect in TRIzol Reagent following standard protocols (we use 12 pellet volumes for early stages and 20 volumes for late stages) and freeze at -80ºC.
- Extract RNA and reverse transcribe using standard protocols. The cDNA obtained can be used for transcriptional analysis by quantitative PCR or microarray (DNase treatment of RNA and subsequent purification using a commercial kit are performed prior to reverse transcription for quantitative PCR analysis).

Comments

- To check that schizonts samples from different parasite lines have been indeed collected at the same stage, we calculate the percentage of burst schizonts as an estimate of the age of the culture. The percentage of burst schizonts is estimated by examination of Giemsa stained smears, counting 200 infected RBCs and scoring them as rings or schizonts, and applying the following formula:

  % burst S = {(number R/IR)/[number S + (number R/IR)]}x100

  S = schizonts
  R = rings
  IR = invasion rate, defined as the increase of parasitemia from one cycle to the following cycle. It is different for each parasite line and culture conditions.

- When transcriptional comparison is performed in schizonts and at a single time point, it is advisable to first determine the number of hours required by different parasite lines to achieve the same percentage of bursting (eg, for the transcriptional comparison of 3D7-A and 3D7-B schizonts, we compare 41-46 h 3D7-A parasites with 39.5-44.5 h 3D7-B parasites) (Cortés et al., 2007).
- This protocol ensures that the transcriptional data obtained for a given time point strictly corresponds to the stage of interest. Samples are collected at the cycle immediately after synchronization (we have experienced that parasites do not require an additional cycle to recover from sorbitol lysis). Therefore, in samples collected at early stages there is no presence of contaminating late forms from the previous cycle (Figure 1).
- When performing transcriptional analysis using microarrays, it is possible to statistically estimate the age of the parasites (Lemieux et al., 2009). We found this method very useful to eliminate the confounding effect of small differences in parasite age (Figure 2 and Supplemental information in Rovira-Graells et al., 2012).
- This protocol has been successfully used in our lab for transcriptional analysis to compare expression between different parasite lines using microarrays, semi-quantitaive, or quantitave PCR, and also for qChIP (Cortés et al., 2007; Crowley et al., 2011; Rovira-Graells et al., 2012).

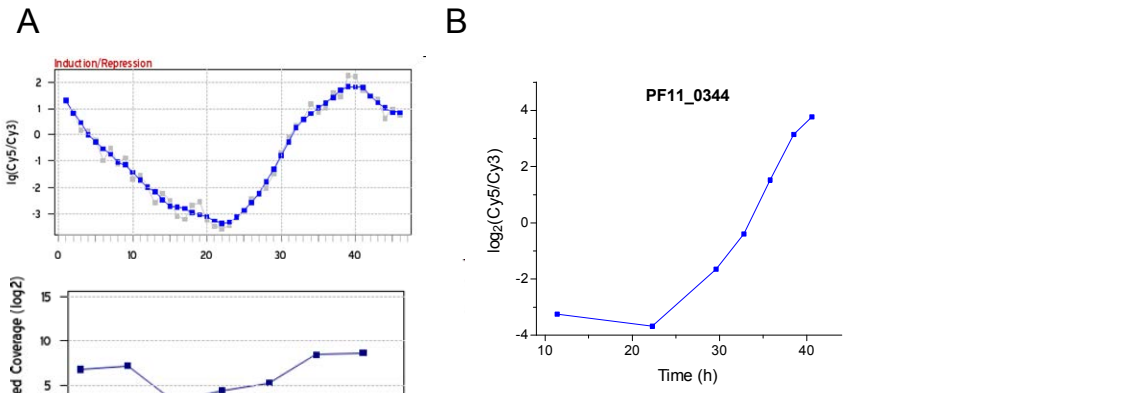

Figure 1. Time course-expression plots for *ama1* (PF11_0344) using different parasite synchronization strategies. **(A)** Commonly used expression plots available in PlasmoDB (www.plasmodb.org), obtained using microarrays (top) or RNA-seq (middle and bottom), show expression of *ama1* both at early and late time points. The expression observed at early time points (rings) is a consequence of the presence of late stage parasites (schizonts) at early time points (even a small proportion of schizonts contribute high levels of schizont-specific transcripts because each schizont has as much RNA as several rings). **(B)** Expression time-course for the same gene (Rovira-Graells et al., 2012) using the protocol described here. No expression of *ama1* is observed in ring-stage parasites.

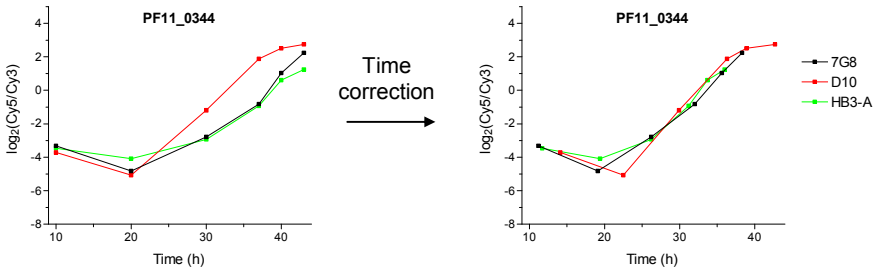

Figure 2. Correction of apparent differences in expression by statistical estimation of parasite age. Expression profiles for *ama1* in three genetically different parasite lines (Rovira-Graells et al., 2012), plotted against experimental times (times at which samples were collected, left) or against statistically-estimated times (right) (Lemieux et al., 2009). Apparent differences in transcript levels (up to 8-fold at some time points) attributable to differences in parasite age (duration of the asexual cycle differs between parasite lines) are eliminated by using estimated parasite age.

References

Cortés, A. et al. Epigenetic silencing of *Plasmodium falciparum* genes linked to erythrocyte invasion. PLoS Pathog. 3: e107 (2007).

Crowley, V.M. et al. Heterochromatin formation in bistable chromatin domains controls the epigenetic repression of clonally variant *Plasmodium falciparum* genes linked to erythrocyte invasion. Mol Microbiol 80: 391-406 (2011).

Lemieux, J. E. et al. Statistical estimation of cell-cycle progression and lineage commitment in *Plasmodium falciparum* reveals a homogeneous pattern of transcription in ex vivo culture. Proc Natl Acad Sci USA 106, 7559-64 (2009).

Rovira-Graells, N. et al. Transcriptional variation in the malaria parasite *Plasmodium falciparum.* Genome Res, 22:925-38 (2012).

IV:E. Enrichment of late-stage infected erythrocytes in 60% Percoll

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Equipment

centrifuge, swing-out rotor, refrigerated

Materials and reagents

mature parasite stages
malaria culture medium (MCM) with the human serum replaced by 10% FBS

60% Percoll solution:
60 mL Percoll
7 mL 10× PBS
33 mL PBS
The solution is enough for 40 tubes of 2.5 mL each, which is enough to enrich 80-mL of culture. Make fresh solutions before each use. Keep all solutions on ice throughout the preparation.

PBS

Procedure

- Wash a mature-stage *Plasmodium falciparum* culture 3 times in MCM with the human serum replaced by 10% FBS and resuspend it to 10% hematocrit in cold MCM with 10% FBS.
- Distribute the culture in centrifuge tubes, 2 mL/tube.
- Using a Pasteur pipette, gently add 2.5 mL of cold 60% Percoll to the bottom of each tube.
- Centrifuge in a swing-out rotor at 1,500 × *g* and 4 °C for 15 min (2,000 rpm in Sorvall or Beckman centrifuge).
- Withdraw carefully the cells at the interphases, pool them, and wash them 3 times with cold PBS (centrifuging each time for 7 to 8 min at 2,000 rpm). The interphases contain trophozoites and schizonts, while rings and uninfected erythrocytes are found in the pellet.

Reference

Wahlgren M, Berzins K, Perlmann P, Björkman A. 1983. Characterization of the humoral immune response in *Plasmodium falciparum* malaria. I. Estimation of antibodies to *P. falciparum* or human erythrocytes by means of microELISA. Clin Exp Immunol 54(1):127-134.

IV:F. Separation of *Plasmodium falciparum* mature stages in Percoll/sorbitol gradients

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Equipment

centrifuge, fixed angle rotor (Sorvall SS-34 or Beckman JA-20)
benchtop centrifuge

Materials and reagents

heparin
PBS (pH 7.2)
Percoll
sorbitol
RPMI 1640
distilled water
malaria culture medium (MCM)
Corex tubes
23 G 0.6-mm ∅ needle
tuberculin syringe

Preparation of gradients (for 4 gradients):

50 mL of Solution H:
(90% Percoll, 5% sorbitol)
45 mL Percoll
5 mL 10× RPMI 1640
2.5 g sorbitol

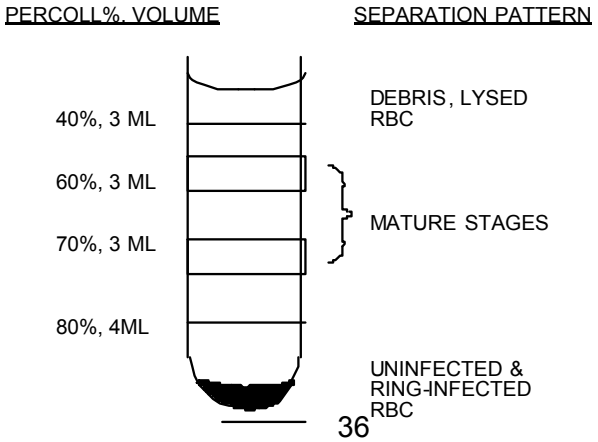
30 mL of Solution L:
(0% Percoll, 5% sorbitol)
27 mL distilled water
3 mL 10× RPMI 1640
1.5 g sorbitol

Prepare gradients:

Volume	Percoll	Solution L	Solution H
15 mL	40%	8.3 mL	6.7 mL
15 mL	60%	5.0 mL	10.0 mL
15 mL	70%	3.3 mL	11.7 mL
20 mL	80%	2.2 mL	17.8 mL

Procedure

- For each gradient, fill a 15-mL Corex tube as indicated below. Using a syringe, and starting with the lightest solution (40%), carefully layer the next denser layer under the previous one:



Separation of parasite stages

- Wash the PRBC and resuspend them in 1 mL of Solution L.
- Add 100 U/mL of heparin and pass the suspension 5 times through a 23 G 0.6-mm Ø needle using a tuberculin syringe (to disrupt rosettes).
- Very carefully overlay the cell suspension on top of the gradient.
- Centrifuge the gradients in a fixed angle rotor at 10,000 rpm for 30 min at 20 °C. Do not use a brake to decelerate.
- Discard the top of the gradient.
- Collect the parasites (floating as described in the diagram above) in a fresh centrifuge tube, no more than 5 mL/tube in a 10-mL tube. Fill the tube with PBS, mix very gently, and spin at 3,000 rpm for 1 min in a benchtop centrifuge.
- Resuspend the PRBC in MCM (see also PARASITES, section I:A).

Reference

Fernandez V, Treutiger CJ, Nash GB, Wahlgren M. 1998. Multiple adhesive phenotypes linked to rosetting binding of erythrocytes in *Plasmodium falciparum* malaria. Infect Immun 66(6):2969-2975.

IV:G. Obtaining free parasites

by ***Denise Mattei***
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Equipment

centrifuge (15-mL tube and microfuge)

Materials and reagents

Plasmion (Fresenius France)
5% solution (w/v) of Gly-Ser (Sigma G3252)
40 mM HEPES
10 mM D-glucose in distilled water

Procedure

- Enrich parasitized red blood cells with Plasmion. Recover them by centrifugation at 2,000 rpm for 2 min. Resuspend the pellet at 37 °C in 1.5 volumes of a 5% solution (w/v) of Gly-Ser made with 40 mM HEPES (pH 7.2) and 10 mM D-glucose in distilled water.
- Incubate at 37 °C for 10 to 30 min.
- Add 5 volumes of culture medium.
- Centrifuge at 1,000 rpm for 3 min. Free parasites are in the supernatant; intact cells are in the pellet. The pellet of intact PRBC can be re-incubated with Gly-Ser.

Reference

Elford B. 1992. Generating viable parasites extra-erythrocytic forms of *P. falciparum*. TDR News 41:11.

IV:H. Obtaining semi-intact cells

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In eukaryotic cells this method extracts soluble cytoplasmic components with the membranes retained.

Equipment

refrigerated microfuge

Materials and reagents

Plasmion (Fresenius France)
washing buffer (WB):
10 mM HEPES (pH 7.2)
15 mM KCl
breaking buffer (BrB):
50 mM HEPES (pH 7.2)
90 mM KCl

Procedure

- Enrich parasitized red blood cells with Plasmion. Wash 1 volume of Plasmion (without centrifugation) 3 times in PBS and resuspend it in the same volume of ice cold washing buffer, WB. For example, if you start with 250 μL of Plasmion, resuspend the pellet in 250 μL of WB.
- Centrifuge the cells in a refrigerated microfuge at 15,000 rpm for 10 min at 4 °C.
- Wash the pellet once in WB and resuspend it in the original volume of WB.
- Incubate it on ice for 10 min. Recover PRBC by centrifugation at 15,000 rpm for 10 min at 4 °C.
- Resuspend the pellet in the original volume of breaking buffer, BrB.
- Centrifuge as above and resuspend the pellet in one tenth of the original volume of BrB. Semipermeable parasites can be used in immunofluorescence studies or as membrane-enriched extracts on Western blots or immunoprecipitations.

Reference

Beckers CJM, Keller DS, Balch WE. 1987. Semi-intact cells permeable to macromolecules: use in reconstitution of protein transport from the endoplasmic reticulum to the Golgi complex. Cell 50(4):523-534.

IV:I. Alanine synchronization of *Plasmodium falciparum*-infected erythrocytes

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Only early trophozoites and noninfected erythrocytes will survive this treatment.

Equipment

centrifuge

Materials and reagents

early trophozoites
noninfected erythrocytes
0.3 M alanine
10 mM HEPES (pH 7.5)
culture medium (RPMI 1640 with 10% heat-inactivated human serum)

Procedure

- Prewarm a solution of 0.3 M alanine, 10 mM HEPES (pH 7.5) at 37 °C.
- Spin down the parasite culture to a pellet (5 min at 2,000 × *g* at 20 °C).
- Resuspend the pellet in 5 volumes of 0.3 M alanine, 10 mM HEPES (pH 7.5).
- Incubate the solution for 3 min at 37 °C.
- Add 10 volumes of culture medium.
- Centrifuge the tube for 5 min at 2,000 × *g* at 20 °C and resuspend the pellet in culture medium at a 5% hematocrit.

Reference

Braun-Breton C, Rosenberry TL, Pereira da Silva L. 1988. Induction of the proteolytic activity of a membrane protein in *Plasmodium falciparum* by phosphatidyl inositol-specific phospholipase C. Nature 332(6163):457-459.

IV:J. Selection of trophozoites by using magnetic cell sorting (MACS)

*by **Anna Leitgeb***
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e-mail: anna.leitgeb@dilaforette.se

Equipment

Vario-MACS magnet (Miltenyi Biotec)
MACS CS-column (Miltenyi Biotec)
3-way stopcock (Miltenyi Biotec)
10-mL syringe
5-mL syringe
0.8-mm needle
0.6-mm needle
50-mL Falcon tubes
microscope
centrifuge

Materials and reagents

4 to 6 mL malaria culture, 5 to 10% parasitemia
PBS
150 mL 2% BSA/PBS
heparin (25000 IU/mL, Lövens)

Preparation

- Connect the 3-way stopcock and the column and place it in the magnet (fig. 1).
- Fill the 10-mL syringe with 2% BSA/PBS and connect it to the left opening of the 3-way stopcock. Open the way between the syringe and the column by closing the way between the syringe and the tap way out (fig. 2). Load the column with 2% BSA/PBS by carefully pushing the connected syringe (fig.3). Fill up to 10 mm above the column matrix. Close the 3-way stopcock totally (fig. 4). Refill the syringe with 2% BSA/PBS and reconnect it to the column. Leave the column for incubation for at least 5 min.
- Preparing the malaria culture: Centrifuge the malaria culture. Resuspend the pellet in 2 mL of 2% BSA/PBS. If using rosetting parasites, add heparin (100 U/mL) and push the culture through a 0.6-mm needle five times. Check in the microscope that all the rosettes are disrupted.
- Cut the top of the cover of a 0.8-mm needle and connect it to the 3-way stopcock (fig. 1). Open the way between the column and the needle and let the excess of loaded 2% BSA/PBS elute. Do not let any air into the column.
- Load the parasites on the top of the column. Open the way between the column and needle. Let the parasites migrate into the column. Close when 1 mm of solution is left. Add some 2% BSA/PBS and let the cells sediment following a quick opening of the tap to let the last cells immigrate. Do not let blood go through the needle. Leave for incubation for 10 min.
- Wash the column by adding 50 mL of 2% BSA/PBS to the top of the column and open between column and the needle. Leave some millimeters of the solution before closing the tap.
- Disconnect the needle from the 3-way stopcock. Disconnect the column from the magnet. Elute the bound material by holding the column upside down over a

Falcon tube and flush through 50 mL of 2% BSA/PBS using the syringe connected to the 3-way stopcock.

- Centrifuge the eluted material at 1,500 rpm for 5 min at room temperature. If using heparin, wash the pellet 5 times in 50 mL of PBS.
- Count the parasitemia.
- The column can be reused immediately. Place the column in the magnet and follow the protocol from the beginning. If reused later, please use the instructions for this procedure delivered together with the columns.

Comments

Approximately 50% of the trophozoites originally found in culture will be an enriched suspension. The following factors have been shown to affect the results:

- The culture should have a parsitemia of 6 to 10%, well synchronized trophozoites.
- The volume of culture used should not be larger than 6 mL or 10^9 cells.
- There should be no (or very few) extracellular parasites in the culture, as they seem to attach better to the matrix.
- A good looking, well-synchronized, culture without extracellular parasites seems to be the most important thing for a good result on the MACS.
- If heparin-free cultures are necessary, rosettes can be disrupted mechanically by passing the culture through the needle without heparin (see above), but make sure all rosettes are disrupted before loading the column.

Reference

Uhlemann AC, Staalsoe T, Klinkert MQ, Hviid L. 2000. Analysis of *Plasmodium falciparum*-infected red blood cells. MACS & more 4(2):7-8. Available from: http://www.miltenyibiotec.com/index.

Figures

Fig 1 – Assembly of the MACS column

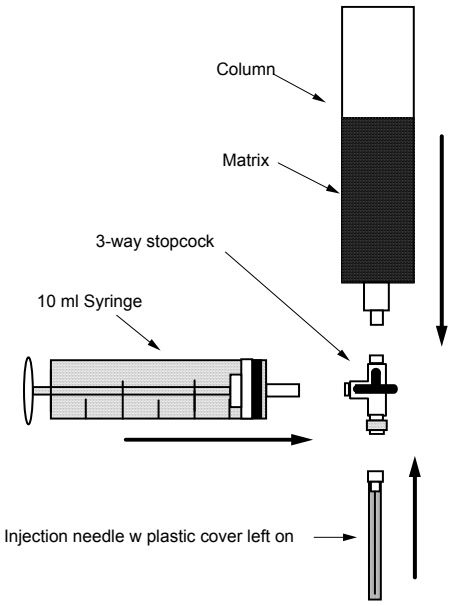


Fig 2.

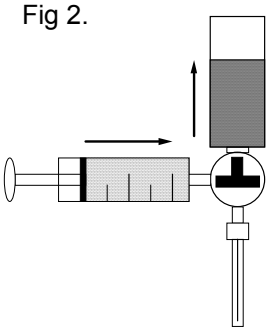


Fig 3.

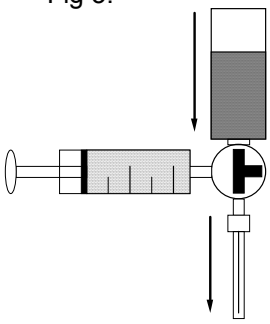
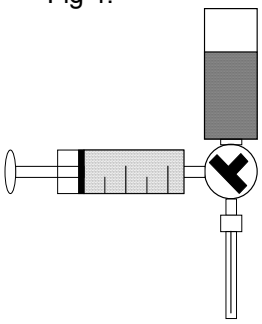


Fig 4.



Data for MACS-column:

Matrix volume	6.3 mL
Reservoir volume	6.5 mL
Maximum capacity total cells	10^9
Maximum capacity retained cells	2×10^8
Rinse volume	60 mL
Wash volume	30 mL
Elution volume	30 mL

IV:K. Isolation of *Plasmodium falciparum*-infected erythrocytes from the placenta

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See PARASITES, section VII:A, page 69

IV:L. Isolation of viable *P. falciparum* merozoites by membrane filtration

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This method can be used to isolate *P. falciparum* merozoites that remain structurally intact and retain their invasive capacity. Merozoites can then be used in erythrocyte invasion assays, invasion inhibition assays, approaches to image merozoite invasion events by microscopy, or other applications. The protocol is developed from Boyle, Wilson, et al., PNAS 2010

Materials and reagents

VarioMACS™ separation magnet system and columns (Miltenyi Biotec).
18G drawing needle.
RPMI-HEPES standard culture medium containing:
- Albumax II (or heat inactivated human serum)
- glutamine
- hypoxanthine
- gentamicin

U-bottom 96 well tissue culture plate (Falcon 3077)
human erythrocytes
10 mL disposable plastic syringe
1.2µm syringe filter (product code 17593; Sartorius)
PBS
E64 (*trans*-Epoxysuccinyl-L-leucylamido(4-guanidino)butane, Sigma product number E3132)

Procedure

1. Preparation of *P. falciparum* parasites

a. Tightly synchronise (within a 6 hour spread of developmental age) 100-150 ml of parasites at 3-5% parasitemia (2-3% hematocrit)
   Recommended synchronisation methods:
   - Heparin synchronization allows for tighter synchronization of parasites, within a known window of invasion (Boyle et al., 2010b). This method is better suited for parasite lines that do not form gametocytes and have a close to 48 hour replication cycle. We routinely use D10-PfPHG parasite line (Wilson et al., 2010) that express GFP. D10 parental, 3D7, W2mef and FCR3 wild-type and knockout lines have also been used for isolation of merozoites. Gametocytes from these lines, if a problem, can usually be reduced by inclusion of a regular sorbitol lysis step (once a week or so).
   - Repeated or double sorbitol synchronising may also result in cultures of sufficient synchronicity; however it will be likely that invasion efficiency of isolated merozoite will be reduced.

b. 100 ml of tightly synchronised culture, 3-5% parasitemia, 2-3% haematocrit will typically result in approximately 4 X 10^8 isolated merozoites (Boyle et al., 2010a).

This amount is sufficient for approximately 60 aliquots of 50 μl volume samples for invasion inhibition assays.

c. When parasites are at early segmented schizont stage (approximately 40-44 hours post-invasion, characterised by a dappled colouration in a Giemsa stained thin smear) pellet cells by centrifugation (2800 rpm, 5 mins) and resuspend in a reduced volume (20 ml) of culture medium or RPMI-HEPES with sodium bicarbonate and proceed to magnet purification of schizonts.

2. Magnet purification of schizonts and E64 treatment

a. Use the VarioMACS™ separation magnet system and CS columns (Miltenyi Biotec) for magnet purification
    - Assemble magnet as per manufacturer's instructions.
    - Wash CS magnet column with 50 ml of RPMI-HEPES, ensuring that no air bubbles remain (it is possible to use larger columns for higher volumes of parasite cultures).
    - Pass schizonts in reduced volume of medium slowly through column (Eg. 20 ml suspension of schizonts as prepared in step 1c). Typical speed used is approximately 1-2 drops per second through the magnet.
    - Wash column while still attached to magnet with 30ml volume of RPMI-HEPES. It is important to wash column extensively to completely remove all uninfected RBCs.
    - Wash column with 5-10 ml of culture medium.
    - Remove column from magnet and elute parasites with 30-40 ml of culture medium. It may be necessary to remove air bubbles at this stage by *gently* filling the column from the bottom with the side syringe.

b. Add E64 to eluted parasites at final concentration of 10 μM
    - Stock solution of E64 at 10 mM in H_2O, filter sterilised, is added at 1/1000 to cultures
        - NOTE: If E64 is added to late trophozoite parasite before clear segmentation, treatment will result in trophozoite death which will subsequently block or damage the filter when isolating merozoites, therefore it is important that the majority of the culture has clearly started to segment prior to the addition of E64.
        - NOTE: It is important to make clear thin smears to accurately judge lifecycle stage. Typically, this is achieved by pelleting 20-30 μL of culture down in a microcentrifuge tube, removing the supernatant and making a smear of the pellet, then staining using Geimsa or other stains.

c. Return purified schizonts to incubator under normal culture conditions for 5-8 hours.

3. Isolation of merozoites

1. Prepare parasites for isolation of merozoites
    - After 5-8 hours of incubation with E64, smear culture pellets, stain slides, and examine smears to ensure that a large proportion of parasites have formed fully mature sacks of merozoites (ideally >70%: the proportion that are fully matured will be dependent on the synchronicity of the original culture) (refer to Figure S1, (Boyle et al., 2010a) http://www.pnas.org/content/107/32/14378/suppl/DCSupplemental)
    - Subsequently, pellet treated schizonts by centrifugation at 1900g for 5 minutes.
    - Remove medium and resuspend culture in the required volume for the assay plus at least 500 μl to account for loss during filtration (40 μl of merozoite is needed per assay well for invasion assays, for 60 well assay plate total volume needed is 2400 μl, so filtrate volume of at least 2900 μl is needed). Since the first 200-300 μl of sample is mainly media with few merozoites, it is recommended to filter in a minimum of 1 ml.
        - NOTE. It has been reported that Albumax can affect filtration of merozoites through membranes (Blackman, 1994). We filter merozoites using RPMI-HEPES culture media supplemented with 10% human serum or RPMI-HEPES incomplete culture media lacking supplemented protein.
    - For isolation of merozoites, prepare the filter by removing the plunger from a 10 ml syringe and attach it to the 1.2 μm filter unit.
    - NOTE: Manufacturer's instructions for 1.2 μm filter indicate that the use of syringe units smaller that 10 ml can result in pressures that may damage the filter unit.
    - Add schizonts to syringe, add plunger, and filter merozoites at an even speed into a pipetting tray.

Reference:

Blackman, M.J. 1994. Purification of *Plasmodium falciparum* merozoites for analysis of the processing of merozoite surface protein-1. *Methods Cell Biol*. 45:213–220.

Boyle, M.J., D.W. Wilson, J.S. Richards, D.T. Riglar, K.K.A. Tetteh, D.J. Conway, S.A. Ralph, J. Baum, and J.G. Beeson. 2010a. Isolation of viable *Plasmodium falciparum* merozoites to define erythrocyte invasion events and advance vaccine and drug development. *Proceedings of the National Academy of Sciences*. 107:14378–14383.

Boyle, M.J., J.S. Richards, P.R. Gilson, W. Chai, and J.G. Beeson. 2010b. Interactions with heparin-like molecules during erythrocyte invasion by *Plasmodium falciparum* merozoites. *Blood*. 115:4559–4568.

Wilson, D.W., B.S. Crabb, and J.G. Beeson. 2010. Development of fluorescent *Plasmodium falciparum* for in vitro growth inhibition assays. *Malar J*. 9:152.

V. Micromanipulation cloning of *Plasmodium falciparum*

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Equipment

inverted microscope (Diaphot 300, Nikon)
micromanipulator system (NT-88, Nikon/NARISHIGE)
micropipette puller (Sutter Instrument, Model P-87)
pipette filling accessories: tubing, needle, syringe, MicroFil (World Precision Instruments)

Materials and reagents

capillaries, Boroglass, outer diameter = 1.0 mm, inner diameter = 0.5 mm (Sutter Instrument)
glass slides
12- or 24-well microplates
0.2-μm syringe filter
culture flasks, 25 cm^2
parasite culture of 5 to 10% parasitemia
malaria culture medium (MCM; see PARASITES, section I:A)
human serum, blood type AB^+ (HS, see PARASITES, section I:A)
human erythrocytes, blood type O^+ (see PARASITES, section I:A)
PBS (pH 7.2)
RPMI 1640
acridine orange (see PARASITES, section III:A)
Acrylease (Stratagene)

Preparation

- Pull pipettes in the micropipette puller. We use program 0 (heat: 392, vel: 100, pressure: 599). Each capillary gives two micropipettes. Break the tip with tweezers to get an inside diameter of 3 to 5 μm. Check in the microscope that the pipette tip edge is blunt.
- Mix MCM with human serum to get 90% MCM/10% HS and filter through a 0.2-μm syringe filter.
- Fill a pipette with filtered 90% MCM/10% HS. Attach the pipette to a tube connected to a needle/syringe. Put the tip of the pipette in filtered 90% MCM/10% HS and fill the tip by pulling the syringe. Hold for 1 to 2 min until the liquid is above the narrow part of the tip. Detach the syringe. Fill the rest of the pipette by using a MicroFil. Coat the outside of the filled micropipettes by dipping the tip in filtered 90% MCM/10% HS for at least 10 min.
- Attach the micropipette to the micromanipulator system.

V:A. Micromanipulation cloning of parasites

- Add 1 mL of filtered 90% MCM/10% HS to well A1 of a microplate. Make sure that the entire surface of the bottom is covered.
- Incubate the plate for 1 h at room temperature.

- Add 1 or 2 mL of 85% MCM/15% HS to the rest of the wells. Use the smaller volume for 24-well plates and the larger for 12-well plates.
- Add 2 to 8 μL of parasite culture to well A1. Dispense and leave it for 15 min to sediment.
- Pick individual erythrocytes with one parasite each and transfer them to the other wells of the microplate, placing only one parasite-infected erythrocyte in each well. If rosetting parasites are picked, strip off the uninfected erythrocytes and make sure that only one parasite is picked.
- Wash erythrocytes three times with RPMI 1640 and add them to all wells (except A1) to get a hematocrit of 1%; 20 μL to 12-well plates and 10 μL to 24-well plates.
- Culture the cells in candle jars as described below in section C below (see also PARASITES, section I:A).

V:B. Micromanipulation cloning of *Plasmodium falciparum* for single-cell RT-PCR

- Siliconize several slides with Acrylease.
- Put a drop of PBS on one of these slides.
- Exchange the filtered 90% MCM/10% HS in the lower part of the micropipette tip with PBS.
- Put 0.2 to 0.3 mL of PBS on another siliconized slide.
- Add 1 to 2 μL of the parasite culture and mix.
- Let the cells sediment for 5 to 10 min.
- On a siliconized slide, dispense 10 μL of PCR mix (see also MOLECULAR BIOLOGY, section V).
- Transfer 1 infected erythrocyte (with 1 parasite) from the parasite-containing drop to the PCR mix drop.
- Pipette the 10-μL drop containing the infected erythrocyte back into the PCR tube and immediately freeze the tube on dry ice.
- Pick uninfected erythrocytes as controls.

V:C. Expansion of *Plasmodium falciparum* clones

- *Day 0:* Cloning (see sections A and B above).
- *Day 3:* Change the medium. Carefully remove the supernatant and add fresh 85% MCM/15% HS. Add 1 mL to 24-well microplates and 2 mL to the 12-well plates.
- *Day 6:* Change the medium and add 1% hematocrit of washed erythrocytes (10 μL to 24-well plates, 20 μL to 12-well microplates).
- *Day 9:* Change the medium.
- *Day 12:* Change the medium and add 1% hematocrit.
- *Day 15:* Change the medium.
- *Day 17:* Change the medium and add 1% hematocrit.
- *Day 19:* Check the clones. Take a drop from the bottom of the well and put it on a slide. Add a drop of acridine orange (see PARASITES, section III:A) and use the microscope to check for parasites. Examine at least 30 fields. If the parasitemia is >1%, move the clone to a 25-cm^2 culture flask with 90% MCM/10% HS and continue to incubate the culture (see PARASITES, section I:A). Change the medium in wells with very low or no detectable parasitemia.

- *Day 21:* Check wells and/or flasks and record the phenotype of the parasites. Discard empty wells and transfer clones with parasites to culture flasks as above (see *Day 19*). When the parasitemia in the flasks is higher than 5% and the parasites are in the ring stage, they can be frozen (see PARASITES, section II).

References

Chen Q, Barragan A, Fernandez V, Sundström A, Schlichtherle M, Sahlén A, Carlson J, Datta S, Wahlgren M. 1998. Identification *of Plasmodium falciparum* erythrocyte membrane protein 1 (PfEMP1) as the rosetting ligand of the malaria parasite *P. falciparum*. J Exp Med 187(1):15-23.

Chen Q, Fernandez V, Sundström A, Schlichtherle M, Datta S, Hagblom P, Wahlgren M. 1998. Developmental selection of *var* gene expression in *Plasmodium falciparum*. Nature 394(6691):392-395.

Fernandez V, Treutiger CJ, Nash GB, Wahlgren M. 1998. Multiple adhesive phenotypes linked to rosetting binding of erythrocytes in *Plasmodium falciparum* malaria. Infect Immun 66(6):2969-2975.

VI. Cytoadhesion and rosetting assays

VI:A. Basic cell media

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CHO and L cell medium, solution for 500 mL:

5.2 g RPMI 1640 (with glutamine, without $NaHCO_3$; Gibco)
3.0 g HEPES (Gibco)
0.4 g geneticin
450 mL distilled water
50 mL fetal calf serum
0.5 mL penicillin/streptomycin (Gibco stock concentrations: penicillin, 10,000 IU/mL; streptomycin, 10,000 μg/mL)
Mix components and filter-sterilize; store at 4 °C for up to one month.

Alternative CHO and L cell medium, solution for 500 mL:

450 mL commercial RPMI 1640 (Gibco with 20 mM HEPES, without L-glutamine)
50 mL fetal calf serum
8 mL geneticin (Gibco stock concentration, 50 mg/mL)
50 mL L-glutamine (Gibco stock concentration, 20 mM, 100×)
0.5 mL penicillin/streptomycin (Gibco stock concentrations: penicillin, 10,000 IU/mL; streptomycin, 10,000 μg/mL)
Mix components and store at 4 °C for up to one month.

HUVEC medium, solution for 500 mL:

5.84 g MCDB-131 (Sigma)
3.0 g HEPES (Gibco)
1.0 g $NaHCO_3$
10 ng/mL (final concentration) epidermal growth factor
1 μg/mL (final concentration) hydrocortisone
0.5 mL penicillin/streptomycin (Gibco stock concentrations: penicillin, 10,000 IU/mL; streptomycin, 10,000 μg/mL)
25 mL fetal calf serum
Mix components and filter-sterilize; store at 4 °C for up to a month.

COS-7 cell medium:

Dulbecco's Modified Eagle Medium (DMEM, Gibco, 41966-029)
10% fetal calf serum
Mix components. Store the medium at 4 °C. Once the serum has been added, use this complete medium within one week.

Binding medium (pH 6.8), solution for 500 mL:

5.2 g RPMI 1640 (with glutamine, without $NaHCO_3$; Gibco)
3.0 g HEPES (Gibco)
450 mL distilled water
50 mL human serum, inactivated, nonimmune AB^+ (10%)
Mix components and set pH to 6.8 immediately before use.

Alternatively, RPMI 1640 and HEPES can be mixed, filter-sterilized, and kept at 4 °C for up to a month. Immediately before use, add 10% human serum and set the pH to 6.8.

10× RPMI, solution for 100 mL:

10.43 g RPMI 1640 (with glutamine, without $NaHCO_3$; Gibco)
6 g HEPES (Gibco)
2 g glucose
100 mL distilled water
Mix components and filter-sterilize. Set pH to 6.8.

VI:B. Thawing melanoma and other cell lines

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Equipment

water bath (37 to 40 °C)
centrifuge (Beckman Allegra 6R)
15-mL Falcon tube (BD Labware)
25-cm^2 culture flask (treated, nonpyrogenic, polystyrene; Corning)
incubator (37 °C, 5% CO_2)

Materials and reagents

appropriate cell medium (see PARASITES, section VI:A)
gelatine (2% gelatine in PBS, warmed for 30 min at 37 °C)

Thawing of cells

- Remove the vial with the frozen cells from the liquid nitrogen container and put it into a water bath at 37 to 40 °C. Thawing should be rapid (40 to 60 s).
- As soon as the ice is melted, remove the vial from the water bath.
- Transfer the contents of the vial to a sterile test tube and add 5 times the amount of pre-warmed complete growth medium appropriate for your cell line dropwise.
- Centrifuge the cell suspension at 300 × *g* for 5 min.
- Discard the supernatant.
- Dilute the cell suspension with an appropriate volume of the recommended culture medium.
- Transfer the suspension to a culture flask and incubate it at 37 °C in 5% CO_2. The flask top should be loose. When cultivating HUVEC cells, the bottom of the flask should be coated with warm gelatine before the cells are added.
- In order to expedite the removal of the protective freezing additive (glycerol or dimethyl sulfoxide) from the culture medium, change the culture medium 24 h after thawing unless stated otherwise in the specific instructions for a particular cell line.

VI:C. Freezing of cell lines

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Equipment

freezing vials (Nunc Cryo Tube Vials; Nalge Nunc International)
15-mL Falcon tube (BD Labware)
Neubauer chamber
microscope (40× lens)
4 °C-refrigerator
−70 °C-freezer
liquid nitrogen tank for long-term storage

Materials and reagents

freezing medium:
- 7 mL RPMI 1640 medium
- 1 mL dimethyl sulfoxide (DMSO)
- 2 mL fetal calf serum (FCS)

EDTA–trypsin solution:
- EDTA, 2 mg/mL
- trypsin, 2.5 mg/mL

appropriate cell medium (see PARASITES, section VI:A)
trypan blue

Freezing of cells

- Prepare the freezing medium.
- Detach the cells from the cell culture flask with 2 mL of EDTA–trypsin solution and resuspend them in 10 mL of fresh medium (see PARASITES, section VI:D).
- Count a sample of cells in trypan blue (1:2 dilution) using a Neubauer chamber.
- Centrifuge the sample at 4 °C (300 × *g* for 5 min) and resuspend the cells with freezing medium at 1×10^6 cells/mL.
- Aliquot the cells into freezing vials and keep them at 4 °C for 2 h.
- Place the vials in −70 °C overnight.
- Place them in liquid nitrogen for long-term storage.

VI:D. Cultivation of CHO, COS, HUVEC, melanoma, and L cells

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Equipment

centrifuge (Beckman Allegra 6R)
15-mL Falcon tube (BD Labware)
25-cm^2 culture flask (treated, nonpyrogenic, polystyrene; Corning)
incubator (37 °C, 5% Co_2)

Materials and reagents

RPMI 1640 medium
EDTA–trypsin solution
    EDTA, 2 mg/mL
    trypsin, 2.5 mg/mL
appropriate medium (see PARASITES, section VI:A)
0.5% gelatine in PBS for HUVEC cells

Passage of cell culture

- Aspirate the old medium from the culture.
- Wash the cells once with RPMI 1640.
- Add 2 mL of EDTA–trypsin.
- Leave the cells for approximately 5 min.
- Shake the flask sharply and check the flask in the inverted microscope to see that the cells have detached.
- Add 10 mL of fresh medium.
- Mix and transfer the suspension to a 15-mL Falcon tube and centrifuge the suspension for 5 min at 300 × *g.*
- Remove the supernatant by aspiration and resuspend the pellet in 1 mL of culture medium.
- Determine the number of cells in a Neubauer chamber.
- Add 5 mL of fresh culture medium to a new 25-cm^2 flask (for HUVEC cells use a gelatine-coated flask, see note below) and add approximately 10^5 cells.
- Incubate the cells at 37 °C in 5% CO_2. The flask top should be loose.

Note: Before seeding the HUVECs, coat the culture flasks with sterile 1% gelatine in PBS for 1 h at 37 °C:

- Add 2 to 3 mL of gelatine in PBS to the cell culture flask.
- Incubate the flasks for 30 min at 37 °C.
- Remove the excess gelatine by aspiration and add cells as above.

VI:E. Formaldehyde-fixation of melanoma cells

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Equipment

24-well microplates
cell culture-treated coverslips (Thermanox plastic coverslip; Nalge Nunc International)
incubator (37 °C, 5% CO_2)
inverted microscope (125×)
microscope (1000×)

Materials and reagents

melanoma C32 cells in culture
appropriate medium (see PARASITES, section VI:A)
EDTA–trypsin solution:
- EDTA, 2 mg/mL
- trypsin, 2.5 mg/mL

RPMI 1640 medium
PBS
1% formaldehyde

Fixation of cells

- Place cell culture coverslip with the cell culture-treated side upwards at the bottom of the wells of a 24-well microplate.
- Add 1 mL of appropriate cell cultivation medium.
- Follow the protocol for subcultivation of cells (see PARASITES, section VI:D), but instead of seeding melanoma cells in cell culture bottles, add approximatelly 50×10^3 cells to each of the wells.
- Leave the melanoma cells to attach to the coverslip in the incubator (37 °C, 5% CO_2) overnight.
- Remove the medium from the melanoma cells and wash twice with PBS.
- Add 0.5 mL of 1% formaldehyde to each well and incubate the cells for 1 h at room temperature.
- Wash them twice with PBS.
- Add PBS and cover the wells; store at 4 °C until use. The maximum storage time is approximately 1 month.

Reference

Schmidt JA, Udeinya IJ, Leech JH, Hay RJ, Aikawa M, Barnwell J, Green I, Miller LH. 1982. *Plasmodium falciparum* malaria. An amelanotic melanoma cell line bears receptors for the knob ligand on infected erythrocytes. J Clin Invest 70(2):379-386.

VI:F. Binding assays to endothelial and melanoma cells

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Equipment

24-well microplates
cell culture-treated coverslips (Thermanox plastic coverslip; Nalge Nunc Int.)
centrifuge (Beckman Allegra 6R)
15-mL Falcon tube (BD Labware)
incubator (37 °C)
glass slides
inverted microscope (125×)
microscope (1000×)

Materials and reagents

melanoma or endothelial cells in culture
malaria culture of 8% parasitemia or higher with a majority of the parasites at trophozoite stage
EDTA–trypsin solution:
EDTA, 2 mg/mL
trypsin, 2.5 mg/mL
RPMI 1640
appropriate medium (see PARASITES, section VI:A)
5% Giemsa solution in phosphate buffer (see PARASITES, section III:B)
1% glutaraldehyde in PBS
glycerol
binding medium (see PARASITES, section VI:A).

Procedure

- Place cell culture coverslips with the cell culture-treated side upwards at the bottom of the wells of a 24-well microplate.
- Add 1 mL of appropriate cell culture medium to each well.
- Follow the protocol for subcultivation of cells (see PARASITES, section VI:D), but instead of seeding cells in culture bottles, add approximatelly 50×10^3 cells to each of the wells with coverslips.
- Leave the cells to attach to the coverslip in the incubator (37 °C, 5% CO_2) overnight. For melanoma cells, please see note at the end of the protocol.
- Inspect cells that have been plated on coverslips; each cell should be separated from other cells for easy reading of binding.
- Centrifuge the malaria culture at 500 × *g* for 5 min at room temperature.
- Wash the culture 3 times (500 × *g*) with binding medium and resuspend in binding medium to a hematocrit of about 2%.
- Aspirate medium from the coverslips in the 24-well plate and rinse once with binding medium. Be careful not to let the coverslip with the attached cells dry.
- Add 0.5 mL of malaria culture suspension to each coverslip.
- Incubate the cells at 37 °C for 60 min, gently rocking by hand every 15 min. Do not use a CO_2 incubator.

- Check the binding of malaria-infected erythrocytes to the cells using an inverted microscope.
- Wash away unbound red cells by dipping the coverslip carefully in each of 3 beakers of binding medium or RPMI 1640.
- Fix the coverslips in 1% glutaraldehyde for 60 min or overnight. Alternatively, fix in 1% methanol for 30 min.
- Rinse coverslips with distilled H_2O and stain with 1% Giemsa for 20 to 30 min.
- After staining, rinse the coverslips with water and let them dry.
- Mount the coverslips on glass slides with glycerol.
- Examine the slides under a microscope (oil, 1,000×) and count 300 cells.
- Calculate the average number of infected erythrocytes bound per target cell.

Note: Melanoma cells can be used fresh or fixed with 1% formaldehyde if fixation insensitive receptors are to be studied, such as CD36 (see PARASITES, section VI:E).

Reference

Marsh K, Marsh WM, Brown J, Whittle HC, Greenwood BM. 1988. *Plasmodium falciparum*: the behaviour of clinical isolates in an in vitro model of infected red blood cell sequestration. Exp Parasitol 65(2):202-208.

VI:G. Selection of cytoadherent parasites by passage over C32 melanoma cells, CHO, HUVEC, or other endothelial cells

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Equipment

centrifuge (Beckman Allegra 6R)
incubator (37 °C)
15-mL Falcon tube (BD Labware)
inverted microscope (125×)

Materials and reagents

malaria culture of 8% parasitemia or higher (e.g., enriched) with a majority of the parasites in trophozoite stage
binding medium (see PARASITES, section VI:A above)
25-cm^2 culture flask covered with melanoma/endothelial cells at approximately 80% confluence
complete malaria culture medium (see PARASITES, section I:A)
human erythrocytes blood type O^+ (see PARASITES, section I:A)

Selection of cytoadherent parasites

- Use malaria cultures with a parasitemia exceeding 8%; lower parasitemias are not worthwhile, because the binding will be too low. The majority of the parasites should be in the trophozoite stage.
- Transfer the malaria culture to a 15-mL Falcon tube and centrifuge at 500 × *g* for 5 min at room temperature.
- Wash the culture 3 times (500 × *g*) in binding medium and resuspend in binding medium at a hematocrit of 2 to 3%.
- Use a 25-cm^2 culture flask covered with melanoma/endothelial cells at approximately 80% confluence.
- Remove growth medium and wash the melanoma/endothelial cells once with binding medium.
- Overlay the cells with the malaria culture suspension.
- Incubate the cells at 37 °C for 1 to 1.5 h. Do not use a CO_2 incubator.
- Resuspend the cells by gently rocking the flask every 15 min.
- Wash the flask gently 3 times with binding medium to remove unbound erythrocytes.
- Check binding of erythrocytes using an inverted microscope.
- Remove binding medium and add complete malaria culture medium and washed fresh human erythrocytes (see PARASITES, section I:A) at 5% hematocrit to the culture flask.
- Transfer the culture to a new flask the next day (after merozoite reinvasion of RBC).
- Continue culturing (see PARASITES, section I).

Reference

Marsh K, Marsh VM, Brown J, Whittle HC, Greenwood BM. 1988. *Plasmodium falciparum:* the behaviour of clinical isolates in an in vitro model of infected red blood cell sequestration. Exp Parasitol 65(2):202-208.

VI:H. Binding of fluorescent receptors heparin, blood group A, and PECAM-1/CD31 to *Plasmodium falciparum*-infected erythrocytes

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Equipment

PCR tubes
centrifuge (500 × *g*)
microcentrifuge (500 × *g*)
glass slides
coverslips
UV light microscope (100× magnification)

Materials and reagents

malaria culture (with parasites in trophozoite stage)
RPMI 1640
ethidium bromide
CAUTION: This chemical is toxic and mutagenic. Wear protective clothing and use only in a chemical fume hood.
for heparin:
fluorescein-conjugated heparin (2 mg/mL, Molecular Probes)
for blood group A:
fluorescein-conjugated blood group A (1 mg/mL A_{tri}-PAA-flu, Syntesome)
for CD31:
recombinant PECAM-1/CD31 (800 µg/mL, R&D Systems)
Alexa Fluor 488 Protein Labeling Kit (Molecular Probes)

Binding of fluorescent receptors

PECAM-1/CD31 is bought unlabelled and must therefore be labelled with a fluorophore before the experiment. Use the Alexa Fluor 488 Protein Labeling Kit to label 400 µg of CD31 in 0.5 mL of PBS. Follow the protocol provided by Molecular Probes.

- Wash the culture (5% hematocrit) twice in RPMI 1640 (500 × *g*) and resuspend to original volume.
- Aliquot 30 µL of washed culture in PCR tubes (0.6 mL). Make one tube for each receptor to be investigated.
- Centrifuge the PCR tubes in a microcentrifuge (500 × *g*), remove the supernatant, and resuspend the cells to a volume of 26 µL with RPMI 1640.
- Add 4 µL of the fluorochrome-conjugated receptor to each PCR tube and mix (regarding CD31 see note below).
- Incubate the cells for 30 min in the dark with resuspension after 15 min.
- Wash the cells 3 times with RPMI 1640 (500 × *g*) and resuspend them to 30 µL in RPMI 1640.

When using heparin-FITC or blood group A-FITC, the fluorescence might be very weak. A possibility is to amplify the signal by using Alexa 488 labelled αFITC (antifluorescein, goat IgG fraction, Alexa Fluor 488 conjugate from Molecular Probes) in a second incubation (dilution 1:8, 30 min at room temperature in the dark with resuspension after

15 min). After this second incubation, wash the cells 3 times as before and continue as described below.

- Resuspend the cells in 30 µL of RPMI 1640 and mix 10 µL of cells with 0.5 to 1 µL of ethidium bromide (1 µL/mL) on a glass slide. Alternatively, to reduce the amount of background, incubate the cells in the PCR tube with ethidium bromide (1 to 2 µL; 1 µg/mL) for 5 to10 s before the last wash.

If fluorescence is weak, ethidium bromide may be skipped altogether as the infected cells can be detected in the microscope by the presence of pigment.

- Add a coverslip and count the fluorescence rate at 100× magnification with oil in a UV-light microscope.

- Calculate the fluorescence rate as = $\frac{\text{number of fluorescent cells}}{\text{total number of late trophozoites}}$

References

Heddini A, Chen Q, Obiero J, Kai O, Fernandez V, Marsh K, Muller WA, Wahlgren M. 2001. Binding of *Plasmodium falciparum*-infected erythrocytes to soluble platelet endothelial cell adhesion molecule-1 (PECAM-1/CD31): frequent recognition by clinical isolates. Am J Trop Med Hyg 65(1):47-51.

Heddini A, Pettersson F, Kai O, Shafi J, Obiero J, Chen Q, Barragan A, Wahlgren M, Marsh K. 2001. Fresh isolates from children with severe *Plasmodium falciparum* malaria bind to multiple receptors. Infect Immun 69(9):5849-5856.

VI:I. Adhesion of *P. falciparum*-infected erythrocytes to immobilized receptors

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Materials and reagents

Marking pens, indelible and suitable for plastic
150mm plastic (polystyrene) tissue culture (petri) dishes
Dako Pen for Immunohistochemistry (Dako Corp) or similar, OR plastic sealing film (eg Parafilm, Nescofilm)
Liquid paraffin oil
Glass coverslips
Phosphate-buffered saline, pH 7.2 OR Tris-buffered saline, pH 7.4
RPMI-HEPES, pH 6.8 - 7.4 (no bicarbonate added)
Bovine serum albumin
Pooled non-immune human serum, OR non-immune AB serum
Glutaraldehyde 2% in PBS
Geimsa stain
Purified receptors
Adhesion medium: RPMI-HEPES pH 6.8 - 7.4, plus EITHER 10% human serum (pooled or AB serum from non-exposed donors) OR 1% BSA

Equipment

Microscope
Class 2 safety cabinet (laminar flow)
Centrifuge

Step-by-step procedure (Beeson et al., 1999)

- On the under-side of a plastic petri dish, mark approximately 10mm diameter circles for the required number of test spots (usually 3 spots for each receptor being tested.) Number or label the circles accordingly.

- Inside the petri dish, on the base, use a Dako pen for immunohistochemistry to copy circles over those drawn above – this creates a circle of plastic that will prevent the parasite suspension being tested (see step 8) from spreading. Alternatively, cut holes in a layer of plastic sealing film using a hole punch and carefully press the plastic film onto the base of the petri dish (see Beeson et al., 1998). Use a blunt instrument (eg. top of a plastic pen) to press the film on completely, taking care around holes to form a good seal with the plastic dish.

- Place 5-10 µl of each receptor, diluted in PBS or TBS, in the centre of each circle.

- Incubate overnight at 4°C in a humid box – ensure the receptor spots do not dry out.

- Before performing the adhesion assay, block non-specific binding by placing a drop or two (50 – 100 µl) of 1% BSA in PBS over each receptor spot. Incubate for at least 30 mins at room temperature.

- Prepare the parasites (in Class 2 cabinet):
  a) Spin down parasites from culture or patient sample at low speed
  b) Wash parasite-RBC pellet once with RPMI-HEPES
  c) remove supernatant and re-suspend pellet in cytoadherence medium (RPMI-HEPES plus either 10% human serum OR 1% BSA) to the required haematocrit, usually 1-5%. We typically use mature pigmented trophozoite-infected RBCs at a parasitemia of 1% or higher.

- Wash the plate with either PBS *OR* RPMI-HEPES by adding 25mls of buffer, agitating and discarding. Repeat once.

- Aspirate, using suction, the remaining buffer from each spot and then add 40 µl of parasite suspension to each well (performed in Class 2 safety cabinet).

- Incubate at 37°C for 30 mins (no agitation).

- Gently wash off unbound cells with RPMI-HEPES pH 6.8 – 7.4, or PBS pH 7.2-7.4
- add 25 ml of buffer carefully to one side of the dish using a pipette. Do not add the buffer directly over receptor spots
- rock gently to remove unbound cells and aspirate supernatant
- wash 4-6 times in total

- Fix bound cells with 2% glutaraldehyde (in PBS) for at least two hours.

- Stain with 10% Giemsa for 10 mins, wash off stain with water, and allow to dry. Do not over stain as it becomes difficult to distinguish infected from uninfected RBCs

- Once dry, cover spots with liquid paraffin oil (or clear vegetable oil) and a glass coverslip

- Count bound infected-RBCs microscopically, expressing counts as bound parasited RBCs/mm^2. Compare adhesion to receptors to that of BSA-coated control spots to account for any non-specific adhesion.

<u>Comments</u>
Steps 6 – 10 should be performed in a Class 2 safety cabinet

Different plastic culture dishes can be used in these assays. We generally use Falcon 1058, but others also work.

Various compounds, serum, or antibodies can be tested for inhibitory activity by incubating parasites with the inhibitor for 5 - 45 minutes prior to step 7 (see Beeson et al., 1998, Reeder et al., 1999)

References:

Beeson, J.G., Brown, G.V., Molyneux, M.E., Mhango, C., Dzinjalamala, F., and Rogerson, S.J. (1999). *Plasmodium falciparum* isolates from infected pregnant women and children are associated with distinct adhesive and antigenic properties. J Infect Dis 180, 464-472.

Beeson, J.G., Chai, W., Rogerson, S.J., Lawson, A.M., and Brown, G.V. (1998). Inhibition of binding of malaria-infected erythrocytes by a tetradecasaccharide fraction from chondroitin sulfate A. Infect Immun 66, 3397-3402.

VI:J. Adhesion of *Plasmodium falciparum*-infected erythrocytes to immobilized hyaluronic acid

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Hyaluronic acid (HA) preparations

HA from bovine vitreous humor (Sigma) is isolated by strong anion exchange chromatography and generally has little or no detectable chondroitin sulfates (Chai et al. 2001). Other sources of HA, but not all, also support adhesion. Some preparations of HA may contain significant amounts of copurified chondroitin sulfates.

Immobilization of HA onto plastic

Immobilization is generally effective with PBS or TBS (pH of 7.2 to 7.4) as the coating buffer, and parasite adhesion is enhanced by including calcium in the coating buffer (and in all other steps). HA effectively adsorbs to the surface of Falcon 1058 plastic dishes (tissue culture treated, Becton–Dickinson).

Optimum conditions for adhesion

- Adhesion of parasites to HA is highest at pH 7.4.
- Include calcium in all steps.
- Use mature pigmented trophozoites in assays rather than schizonts.
- Fixation of bound parasites with glutaraldehyde (see PARASITES, section VI:A) may decrease observed adhesion to HA. It may be necessary to count numbers of cells bound to HA prior to fixation.

Materials and reagents

hyauronate lyase (hyaluronidase) from *Streptomyces hyalurolyticus*
hyaluronic acid isolated from bovine vitreous humor
phosphate-buffered saline

Step-by-step procedure for preparation and use of lyase-treated hyaluronic acid as a control in adhesion assays

- Incubate HA (1 mg/mL) with and without hyaluronate lyase (10 to 20 TRU/mL) in PBS, at 60 °C for 4 h or overnight.
- Stop incubation by boiling HA samples for 10 min at 100 °C. Cool samples and centrifuge them at high speed to remove any insoluble aggregates.
- Make aliquots of treated and untreated HA samples and store them at −20 °C.
- To measure parasite adhesion to HA, dilute lyase-treated and untreated HA samples 1:10 (i.e., a final HA concentration of 100 μg/mL) in PBS and coat plastic surfaces with it as described above (see PARASITES, section VI:A).
- Perform adhesion assay as described above (see PARASITES, section VI:A).
- Interpretation: Adhesion of parasites to nonlyase-treated HA but not to lyase-treated HA indicates specific adhesion to HA.

References

Beeson JG, Rogerson SJ, Cooke BM, Reeder JC, Chai W, Lawson AM, Molyneux ME, Brown GV. 2000. Adhesion of *Plasmodium falciparum*-infected erythrocytes to hyaluronic acid in placental malaria. Nat Med 6(1):86-90. [see comments.].

Chai W, Beeson JG, Kogelberg H, Brown GV, Lawson AM. 2001. Inhibition of adhesion of *Plasmodium falciparum*-infected erythrocytes by structurally defined hyaluronic acid dodecasaccharides. Infect Immun 69(1):420-425.

VI:K. Binding of *Plasmodium falciparum*-infected erythrocytes to placental tissue sections

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See PARASITES, section VII:B, page 71

VI:L. Enrichment of rosetting parasites using Ficoll–Isopaque (Pharmacia)

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Equipment

immufuge blood centrifuge

Materials and reagents

Ficoll–Isopaque (FIP) (Pharmacia)
RPMI 1640
MCM with 10% heat-inactivated serum

Protocol

- Use 2 mL of culture per tube, mature and rosetting parasites, with a 5 to 10% parasitemia and layer it very carefully on 2 mL of sterile, ice-cold Ficoll–Isopaque (FIP).
- Centrifuge the tube in the immufuge blood centrifuge for 12 s on the high setting at room temperature.
- Quickly suck up the pellet using a Pasteur pipette.
- Wash the pellet 3 times using RPMI 1640.
- Subculture (see PARASITES, section I) using fresh, washed erythrocytes and MCM with 10% heat-inactivated serum.

Reference

Udomsangpetch R, Wåhlin B, Carlson J, Berzins K, Torii M, Aikawa M, Perlmann P, Wahlgren M. 1989. *Plasmodium falciparum*-infected erythrocytes form spontaneous erythrocyte rosettes. J Exp Med 169(5):1835-1840.

VI:M. Reversion of rosettes

*by **Anna Leitgeb***
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Equipment

UV-light microscope (40×)

Materials and reagents

96-well microtiter plate
Parafilm
acridine orange

Procedure

- Keep the *Plasmodium falciparum* culture under standard conditions with 10% normal serum added to the buffered medium (see PARASITES, section I:A).
- Perform the studies on cultures with 5% hematocrit, 4 to 8% parasitemia at late stage, and >50% rosetting rate.
- Mix an aliquot of 25 to 50 μL of the rosetting *P. falciparum* culture (1:1) with various dilutions (e.g., 1:5) of sera to be tested. Also prepare a control using nonimmune human sera or MCM (see PARASITES, section I:A).
- Incubate the cultures at 37 °C for 60 min in a 96-well microtiter plate. Cover the plate with Parafilm.
- Use two separate aliquots from each well, mix each with a small amount of acridine orange, and mount them on glass slides. Count 25 consecutive fields of vision per slide using a 40× lens and incident UV light (2 slides/well, approximately 50 fields). Count fields of vision diagonally over the slide, from one corner to the other, in order to compensate for a possible uneven distribution of rosettes on the slide. If the cell concentration is low, it may be necessary to count in both diagonal directions.

Comments

Infected erythrocytes (except ring forms) within a rosette and infected erythrocytes not connected to a rosette are scored separately.

Infected erythrocytes that have bound two or more noninfected cells are scored as rosettes. If the rosette contains more than one infected erythrocyte, the cells are scored separately.

Ring forms within a rosette are not counted.

Late-stage (trophozoite and schizont, but not ring forms) infected erythrocytes are scored and added to the total number of late infected erythrocytes.

Since it can be hard to tell the difference between a ring form and an early trophozoite, only parasites with a diameter that covers at least one-third of the diameter of the erythrocyte are counted.

The number of infected erythrocytes in large rosettes (where it can be difficult to determine the exact number of infected cells) are counted as 3.

If it is difficult to determine the number of cells (usually 2 or 3) bound to an infected erythrocyte, the coverslip can be pressed lightly (using a pencil) to induce a slight movement. This is important, because quite often it is found that only 1 erythrocyte is actually bound to the infected cell, when others that may appear to be are not.

Use two controls, one before counting the samples and one after.

Reference
Carlson J, Holmquist G, Taylor DW, Perlmann P, Wahlgren M. 1990. Antibodies to a histidine-rich protein (PfHRP1) disrupt spontaneously formed *Plasmodium falciparum* erythrocyte rosettes. Proc Natl Acad Sci USA 87(7):2511-2515.

VII. Placental malaria

VII:A. Isolation of *Plasmodium falciparum*-infected erythrocytes from the placenta

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Materials and Reagents

50 ml plastic tubes
scalpel or scissors, forceps
Phosphate buffered saline with 50mM EDTA

Equipment

Microscope
Class 2 safety cabinet (laminar flow)
Centrifuge
Rotating wheel or tube roller
Protective eye wear and face mask

Step-by-step procedure (Beeson et al., 1999)

- Collect placenta as soon as possible after delivery, and determine whether it is infected by performing a blood smear. Orientate the placenta so the maternal side is facing up. Clean the surface with PBS if necessary.

- From apparently healthy areas of the maternal surface, cut several pieces of placental tissue (around 1.5-2 cm in each dimension, up to 8 cm^3) from different areas using either a scalpel or scissors and forceps. Immediately put cut pieces of placental tissue into 50ml tubes containing PBS with 50mM EDTA. Several pieces can be placed into a single tube, but the placental tissue should occupy no more than one third of the volume. Protective eyewear and a face mask should be worn during this procedure.

- Mix tubes containing placental tissue on a rotating wheel or tube roller for 1 hour at room temperature. To increase yield of placental blood and parasites, placental tissue can be cut into smaller pieces or finely chopped after 30 mins, and the incubation then continued for a further 30 mins. Doing this does not appear to significantly influence the parasitemia or parasite phenotypes of the preparation.

- Stop incubation, then compress the placental tissue with a blunt instrument to further remove any blood and parasites. Let tubes stand for one minute to allow tissue pieces and debri to settle.

- Collect the supernatant then centrifuge and wash pellet 3 times with PBS.

- Prepare a thin smear and determine the parasitemia of the preparation.

- Leukocytes can be depleted by passing a suspension of the placental blood over a cellulose column, or over Percoll density gradients.

- If necessary, the parasitemia can be enriched by passage over Percoll density gradients, gelatin flotation, or magnet purification on MACS columns, as described elsewhere

References:

Beeson, J.G., Brown, G.V., Molyneux, M.E., Mhango, C., Dzinjalamala, F., and Rogerson, S.J. (1999). *Plasmodium falciparum* isolates from infected pregnant women and children are associated with distinct adhesive and antigenic properties. J Infect Dis 180, 464-472.

Beeson, J.G., Rogerson, S.J., Cooke, B.M., Reeder, J.C., Chai, W., Lawson, A.M., Molyneux, M.E., and Brown, G.V. (2000). Adhesion of Plasmodium falciparum-infected erythrocytes to hyaluronic acid in placental malaria. Nature Med 6, 86-90.

VII:B. Binding of *Plasmodium falciparum*-infected erythrocytes to placental tissue sections

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Equipment

cryostat at −20 °C

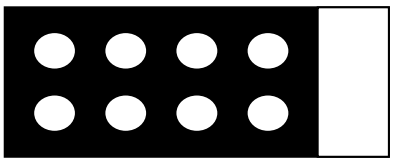
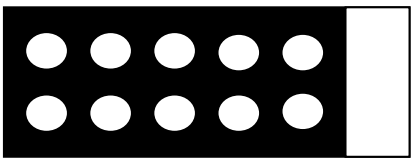
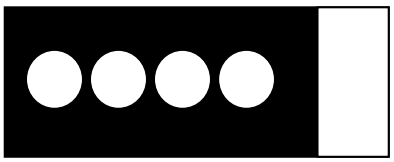
Materials and reagents

binding medium:
5.2 g RPMI powder
3.0 g HEPES
500 mL distilled H_2O

liquid nitrogen
Polymorphprep (Axis-shield)
methanol
distilled H_2O
hyaluronidase: 10 µg/mL enzyme in PBS (pH 7.2)
Streptomyces hyaluronate lyase: 25-50 U/mL in PBS (pH 7.2)
chondroitinase: 0.5 units/mL in Tris–HCl (pH 8.6)
heparinase: 0.5 units/mL in PBS
PBS
slides with 8 wells of 8 mm, 10 wells of 6 mm or 4 wells of 9 mm (Novakemi AB)
coverslips
50-mL Falcon tubes

Placenta sections:

- Cut fresh placental tissue into small cubes (≈ 1 × 1 cm) and freeze immediately in liquid nitrogen. Transfer them later to −70 °C for storage.
- Adapt the placental tissue cubes to −20 °C for 1 h before use.
- Cut 5-µm thick sections with a cryostat at −20 °C.
- Transfer the sections to 8- or 10- well slides (see below).
- Keep the slides with sections at −70 °C. The sections should not be older than 1 week when used in experiments.
- For use, remove a slide from −70 °C, transfer it immediately into cold PBS, and keep it in PBS until use.

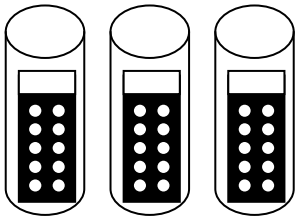




Preparation of PRBC:

- PRBC from fresh placental isolates are isolated from the placenta using the method of Beeson et al (see section VII.A) with PBS-EDTA. Leukocytes are depleted by passing the suspension of eluted material on polymorphprep.
- Enrich the infected cells for late trophozoites in a Percoll-gradient or by MACS purification (Optional for freshly eluted placental isolates).
- Keep the cells as packed cells on ice after purification.

Binding of PRBC to placenta sections:

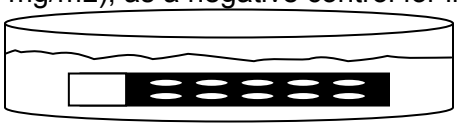
- Resuspend 1-2 µL or 5 µL of packed cells in 23-24 µL or 45 µL of binding medium (for 6-8 mm wells versus 9 mm wells) obtaining a hematocrite of 10%.
- Add those 25 µL or 50 µL to one well containing a placental section, forming a drop on top of the section.
- Incubate the section for 1 h at 37 °C.
- Tip off the drop carefully.
- Wash the section 3 times for 5 min in binding medium: Use three 50-mL Falcon tubes with 40 mL of binding medium each; let the slide stand in each for 5 min.
- Fix the sections on a slide for 15 s in methanol.
- Let them air dry.
- Stain the sections with 5% Giemsa for 30 min (overlay sections with a drop of Giemsa stain).
- Wash away the Giemsa stain with distilled H_2O.
- Put a coverslip over the sections.



Binding of PRBC to placenta sections under the influence of inhibitor molecules (e.g. CSA, IgG):

- Incubate the placenta sections with 25 µL or 50 µL of binding medium or PBS containing the inhibitor for 1 h at 37 °C.
- Wash the sections for 3 min with PBS in a petri dish placed on a slow-moving shaker (see below).
- AND/OR: Resuspend PRBC in binding medium containing the inhibitor and incubate them for 1 h at 37 °C.

Perform the binding assay as described above.
Include at least 2 wells with a positive control (i.e., with no inhibitor present) on each slide to control the quality of the experiment.
Also assess the adhesion of each isolate in the presence of a decoy inhibitor molecule e.g BSA (1-5 mg/mL), as a negative control for inhibition.



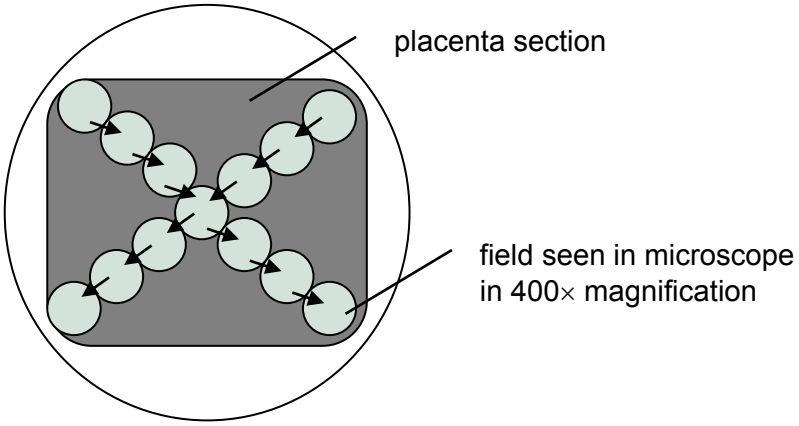
Enzyme treatment of placenta sections:

- Use a volume of 25 μL or 50 μL on each section:
  - hyaluronidase: 10 μg/mL of enzyme in PBS (pH 7.2); incubate at 37 °C for 45 min.
  - *Streptomyces* hyaluronate lyase 25-50 U/mL in PBS (pH 7.2); incubate 1 hr at 37 °C.
  - chondroitinase: 0.5 units/mL in Tris–HCl (pH 8.6); incubate at 37 °C for 10 to 30 min.
  - heparinase: 0.5 units/mL in PBS, incubate at 37 °C for 30 min.
- Wash the sections for 3 min with PBS in petri dishes.
- Include at least 2 wells with a positive control (i.e., no enzyme treatment) on each slide to control the quality of the experiment.

Perform the binding assay as described above.

Counting of bound PRBC in the microscope:

- Count from upper left corner to lower right corner of the section, field next to field at 400× magnification.
- Repeat from upper right corner to lower left corner.
- Determine bound PRBC/field, calculate to PRBC/mm^2. Count at least 30 high power fields.
- The level of adhesion or inhibition in adhesion after treatment is expressed as percentage of the positive control. Cut-off is set to the mean inhibition level obtained with the BSA controls + 2 SD.



Reference
Flick K, Scholander C, Chen Q, Fernandez V, Pouvelle B, Gysin J, Wahlgren M. 2001. Role of nonimmune IgG bound to PfEMP1 in placental malaria. Science 293(5537): 2098-2100.

Rasti N, Namusoke F, Chene A, Chen Q, Staalsoe T, Klinkert MQ, Mirembe F, Kironde F, Wahlgren M. 2006. Nonimmune immunoglobulin binding and multiple adhesion characterize *Plasmodium falciparum*-infected erythrocytes of placental origin.Proc Natl Acad Sci U S A. 12;103(37):13795-800.

VIII. Detection of antibodies to the infected erythrocyte surface

VIII:A. Reversion of rosettes

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See PARASITES, section VI:M, p. 67

VIII:B. Agglutination assay using purified trophozoite-infected erythrocytes
Measurement of antibodies to *P.falciparum* variant antigens on the surface of infected erythrocytes

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Materials and reagents

Phosphate-buffered saline
Microscope slide
Glass coverslips
Mounting medium (eg Depex)
96-well microtitre plates, Epindorf tubes (1.5 ml), or similar

Step-by-Step procedure

- Isolate trophozoite-infected RBCs (not schizonts) from culture using Percoll density gradients, gelatin, magnetic MACS column, or other methods, described elsewhere.

- After harvesting parasites, carefully and gently wash three times with warm PBS

- Make a smear, stain with Geimsa, and examine parasite preparation – check parasite-infected RBCs are intact. Aim for 30% parasitaemia or higher

- Incubate parasites with human serum diluted 1/10, for 45 mins on a rotating wheel, room temperature.
- This is best done by putting 2.5ul aliquots of each test serum into individual wells of a 96-well microtitre plate (or using Epindorf tubes). Then add 22.5 ul of parasite suspension (at a 2% haematocrit) to each well containing test serum
- Always include a negative control serum
- Usually parasites harvested from 25 ml of culture (5-10% parasitaemia, 3% haematocrit) can be resuspended in 0.5-1.0ml of PBS

- Following incubation, pipette out 10ul of the suspension and spread gently onto a glass slide making a circle of about 1.5cm diameter, using a plastic pipette tip. Make 2 ‘smears’ for each samples (on the same or separate slides). [Alternatively, to examine cells as ‘wet preps’, place a coverslip over a 10ul drop (do not smear out) and examine by microscopy (step 7). In this case, infected

RBCs will need to be stained with either ethidium bromide 10ug/ml or SYBR Green 1:5000]

- Allow to dry (5 minutes), fix smears in fresh methanol, stain for 5-10 mins with 10% Geimsa (do not over-stain). Once dry, coverslip slides using a permanent mounting medium (eg. Depex). Slides can the be stored (protected from light) for long periods and examined or re-examined at any time.

- Examine both smears for each sample, by light microscopy
    a) initially scan for agglutinates using 10X objective
    b) examine any agglutinates at 40X
    c) only agglutinates of 5 infected cells or more are considered positive
    d) record the percentage of parasitised RBCs in agglutinates and the size of the largest agglutinate observed

Comments

- Only count agglutinates of parasitised RBCs – don't count agglutinates of parasites that have broken out of the RBC membrane (which frequently and easily agglutinate). Make sure you can see an intact RBC membrane around cells in agglutinates
- Watch out for agglutinates of cells with debri – agglutinated cells must be in contact with each other
- Do not count linear agglutinates – cells lined up in a row are rarely true agglutinates. Find an area where cells are evenly distributed

VIII.C. Mixed Agglutination Assay

Measurement of cross-reactive antibodies to *P. falciparum* antigens on the surface of infected erythrocytes

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Materials and reagents

Parasite dyes (2 different colours needed) - Ethidium bromide, DAPI, or SybrGreen
Phosphate-buffered saline
Microscope slides
Glass coverslips
96-well microtitre plates, Epindorf tubes (1.5 ml), or similar

Step-by-Step procedure

- Isolate trophozoite-infected RBCs (not schizonts) from culture. Use parasites at 5-10% parasitemia

- After harvesting parasites, carefully and gently wash three times with warm PBS

- Stain parasitized RBCs. Mixed agglutination assay is performed with two different parasite lines, each labeled with a different fluorochrome. Separately stain each parasite line with a different dye (Eg. one with ethidium bromide and one with DAPI), then wash parasites 3 times with PBS. To stain parasites, incubate parasitized RBCs with the dye for 30mins at room temp at appropriate concentration, in darkness

- Mix equal amounts of the two different parasite lines.
    e) The parasitemias of each line should be roughly equal. It may be necessary to adjust the parasitemias with uninfected RBCs to get them equal
    f) Resuspend parasite mixture to 5% haematocrit in PBS

- Incubate the parasite suspension with human test sera diluted 1/10, for 1 hour on a rotating wheel, room temperature.
- This is best done by putting 2.5ul aliquots of each test serum into individual wells of a 96-well microtitre plate (or using Ependorf tubes). Then add 22.5 ul of parasite suspension (at a 3-5% haematocrit) to each well containing test serum
- Always include a negative control serum

- Following incubation, resuspend cells if pellet is present, pipette out 10ul of the suspension, and mount a coverslip. Make 2 preparations for each samples (on the same or separate slides). Store slides in a dark humid box until examination.

- Examine both smears for each sample, by combined light and fluorescent microscopy
    a) initially scan for agglutinates using 10X objective
    b) examine any agglutinates at 40X
    c) only agglutinates of 3 infected cells or more are considered positive. Need to see 5 or more agglutinates to consider a sample as positve

d) Count agglutinates and record the number of single colour (EtBr or DAPI) and mixed colour agglutinates – calculate the proportion of agglutinates that were mixed or formed by each isolate
e) Among mixed agglutinates, count proportion that were formed by each isolate

Comments

- Only count agglutinates of parasitised RBCs – do not count agglutinates of parasites that appear to have broken out of the RBC membrane (which frequently and easily agglutinate). Make sure you can see an intact RBC membrane around cells in agglutinates
- Watch out for agglutinates of cells with debri – agglutinated cells must be in contact with each other
- Do not count linear agglutinates – cells lined up in a row are rarely true agglutinates. Find an area where cells are evenly distributed

Examples of mixed agglutinates can be seen here:
http://iai.asm.org/content/78/5/1963.long
http://iai.asm.org/content/78/5/1963/F4.large.jpg

Reference:

Hommel, M., Elliott, S.R., Soma, V., Kelly, G., Fowkes, F.J., Chesson, J.M., Duffy, M.F., Bockhorst, J., Avril, M., Mueller, I., et al. (2010). Evaluating the antigenic diversity of placental binding *Plasmodium falciparum* variants and the antibody repertoire among pregnant women. Infect Immun 78, 1963–1978.

Beeson JG, Mann EJ, Byrne TJ, et al. Antigenic differences and conservation among placental *Plasmodium falciparum*-infected erythrocytes and acquisition of variant-specific and cross-reactive antibodies (2006). J Infect Dis 2006;193:721-730

Newbold, C.I., Pinches, R., Roberts, D.J., and Marsh, K. (1992). *Plasmodium falciparum*: The human agglutinating antibody response to the infected red cell surface is predominantly variant specific. Exp Parasitol 75, 281-292.

VIII:D. Serum micro-agglutination of infected erytrocytes

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Equipment

UV light microscope
rotator

Materials and reagents

RPMI 1640/HEPES/sodium bicarbonate/ gentamycin solution (MCM; see PARASITES, section I:A)
acridine orange (see PARASITES, section III:A)
polystyrene round-bottomed tubes

Procedure

- Culture parasitized red blood cells (PRBC) to the late trophozoite and/or early schizont stage
- Wash the cells 3 times in MCM; resuspend the PRBC at 20% hematocrit.
- Dispense aliquots of 25 μL in prelabelled polystyrene round-bottomed tubes.
- Add 25 μL of serum to the parasite suspension (1:2 dilution). For a 1:5 dilution serum test, add prediluted serum at 1:2.5 in MCM.
- Incubate the PRBC and serum mixture at 37 °C for 1 h with constant rotation.
- Mount an aliquot from each polystyrene tube, mix with a small amount of acridine orange on a glass slide, and count 50 consecutive fields of vision diagonally using a 40× lens and incident UV-light microscopy.
- Always use negative and positive control sera along with the test sera.

Interpretation

- Score the assay as negative when no agglutinates of 4 or more PRBC are detected in the examined slide.
- Use the following semiquantitative scoring scale for agglutination analysis:
  - (−) for no agglutinate of 4 or more PRBC
  - (1+) for 1 to 5 agglutinates of 4 to 10 PRBC
  - (2+) for >5 agglutinates of 4 to 10 PRBC or 1 to 5 agglutinates of 11 to 20 PRBC
  - (3+) for >5 agglutinates of 11 to 20 PRBC or 1 to 5 agglutinates of >20 PRBC
  - (4+) for >5 agglutinates of >20 PRBC
  - (UA) for unspecific agglutination
- When using rosetting strains, note the presence or absence of rosettes.

References

Aguiar JC, Albrecht GR, Cegielski P, Greenwood BM, Jensen JB, Lallinger G, Martinez A, McGregor IA, Minjas JN, Neequaye J, et al. 1992. Agglutination of *Plasmodium falciparum*-infected erythrocytes from east and west African isolates by human sera from distant geographic regions. Am J Trop Med Hyg 47(5):621-632.

Barragan A, Kremsner PG, Weiss W, Wahlgren M, Carlson J. 1998. Age-related buildup of humoral immunity against epitopes for rosette formation and agglutination in African areas of malaria endemicity. Infect Immun 66(10):4783-4787.

Marsh K, Howard RJ. 1986. Antigens induced on erythrocytes by *P. falciparum*: expression of diverse and conserved determinants. Science 231(4734):150-153.

VIII:E. Flow cytometry detection of surface antigens on fresh, unfixed red blood cells infected with *Plasmodium falciparum*

Method developed by by ***Helene Jouin***

The indirect immunofluorescence Labeling is performed as usual on living cells in suspension, and parasitized erythrocytes are selected by gating the fluorescence intensity of either Hoechst 33 342 or thiazole orange (TO) dyes, which reflect the erythrocyte content of parasitic DNA or RNA. TO allows identification of only mature stages of the parasite. Nevertheless, since we have not observed surface immunostaining of the young stages of the parasite when using Hoechst, TO is a good reagent because it allows performing the flow cytometric analysis with a single laser analyser.

Materials and reagents

Hoechst 33 342 (Sigma)
thiazole orange (TO) (Retic-COUNT, BD Immunocytometry Systems)
FACSFlow solution (BD Immunocytometry Systems) containing 2% fetal calf serum

General procedures for DNA or RNA staining, surface immunofluorescence staining, and flow cytometry analysis

When Hoechst is used, the DNA staining is performed first, followed by surface immunofluorescence staining (using FITC or phycoerythrin conjugated second antibody). It is necessary to use two lasers because of the differing excitatory wavelengths of the two fluorochromes.

When TO is used, surface immunofluorescence staining is performed before the RNA staining. The samples can be analysed on a single laser (488 nm). Since TO fluorescence is read using the FITC channel, FITC conjugates cannot be employed for surface immunofluorescence.

Intraerythrocytic parasite DNA staining with Hoechst 33 342 (before surface immunofluorescence staining)

- Dissolve Hoechst 33 342 at 1 mg/mL of distilled water.
- Add the solution directly to the culture medium at a final concentration of 20 μg/mL.
- Incubate the culture for 30 min at 37 °C in the dark.
- Wash the culture in PBS and then perform the immunofluorescence staining.

Intraerythrocytic parasite DNA staining with TO (after immunofluorescence staining)

- Resuspend the washed immunofluorescent stained parasitized red blood cells directly in 1 mL of a solution of TO.
- Keep in the dark for 30 min prior to flow cytometric analysis.

Surface immunofluorescence staining

- Resuspend a pellet of 5×10^6 parasitized red blood cells in 100 μL of immune serum diluted 1:50 in FACSFlow solution containing 2% fetal calf serum.
- Incubate the pellet at room temperature for 30 min.
- Wash it 3 times with FACSFlow-FCS.
- Resuspend the pellet in the appropriate fluorescent-conjugated antibody.

- Incubate it at room temperature for 30 min.
- Wash it twice with FACSFlow-FCS.
- Resuspend it in 1 mL of FACSFlow.

Flow cytometric analysis

Parasitized red blood cells that have been stained with Hoechst 33 342 must be analysed on a cell sorter equipped with two lasers: a UV laser tuned to 320 nm for the excitation of the Hoechst dye and a second laser tuned to 488 nm for the excitation of FITC and phycoerythrin.

Parasitized red blood cells which have been stained with TO and phycoerythrin conjugates can be analysed on a standard flow cytometer equipped with only one laser tuned to 488 nm. The TO emission is detected in the FITC channel. The color compensation between TO and phycoerythrin fluorescences is set up using a control sample (without surface immunofluorescence staining).

Erythrocytes are gated on the basis of their forward scatter and side scatter signals using logarithmic scales. Parasitized erythrocytes are gated on the basis of their positive staining with Hoechst 33 342 or TO. All fluorescence parameters are recorded with logarithmic amplification. Process list mode data from 10,000 gated cells.

Reference

Jouin H, Goguet de la Salmoniere YO, Behr C, Huyin Qan Dat M, Michel JC, Sarthou JL, Pereira da Silva L, Dubois P. 1995. Flow cytometry detection of surface antigens on fresh, unfixed red blood cells infected by *Plasmodium falciparum*. J Immunol Methods 179(1):1-12.

VIII:F. Analysis by flow cytometry of antibodies to variant surface antigens expressed by *P. falciparum*-infected erythrocytes

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Materials and reagents

Phosphate buffered saline (PBS) OR RPMI-HEPES
Casein (filtered)
Ethidium bromide 1mg/ml (filtered to remove aggregates) OR Fetal calf serum (FCS) (heat-inactivated and filtered to remove aggregates)
Secondary antibody - Anti-human IgG (affinity purified). Eg. rabbit anti-human IgG
Tertiary antibody – Alexa-conjugated antibody against the IgG species of the secondary antibody (affinity purified) Eg. Anti-rabbit IgG; or Alexa-Fluor 488-conjugated
U-bottom 96 well microtitre plates

Procedure

Buffers to use in the assay:
For all incubations and washes, either RPMI-HEPES or PBS can be used, with either 0.1% casein or 1% FCS added

- Use parasitised red blood cells (PRBCs) from in vitro culture at 1-5% parasitaemia, synchronous at mid-late stage pigmented trophozoites (not schizonts)
- Wash PRBCs three times in PBS with 0.1% casein or 1% FCS prior to using in the assay
- Pre-coat the required number of wells of a U-bottom 96 well microtitre plate with 0.1% casein or 1% FCS in PBS for 30 mins, room temperature, to block non-specific binding. This reduces cell loss in the assay. Drain wells after incubation
- Aliquot serum or plasma samples (heat-inactivated) to be tested into 96 well plate
- Resuspend PRBCs to 1-2 x 10^7 cells/ml (0.1-0.2% haematocrit)
- Add 50 ul PRBC suspension per well containing test serum to achieve desired serum concentration (usually 1/10 or 1/20)
- Incubate 30 mins at room temperature, then wash 3 times
- Add 50 ul of secondary antibody (Eg. rabbit anti-human IgG (Fc. Sp.))
- Incubate 30 mins at RT, then wash 3 times
- Add 50 ul of fluorescent tertiary antibody (Eg. Alexa-Fluor 488-conjugated antibody) diluted in buffer containing ethidium bromide 10-20 ug/ml

- Incubate 30 mins at RT **in the dark**; wash 3 times

- Resuspend cells in 200 ul of buffer and transfer to flow cytometry tubes

Analysis by flow cytometry

- Gate RBC population based on FSC and SSC
- Plot cells by fluorescence in channel 1 (for Alexa-Fluor488 or FITC) against fluorescence in channel 2 (for ethidium bromide). There should be a clear separation of ethidium bromide positive cells (PRBCs) from negative cells (RBCs)
- Acquisition time should be sufficient to count 500-1000 ethidium bromide positive cells
- For the ethidium bromide negative and positive populations, determine the mean fluorescence intensity in channel 1 (antibody binding) of the cell population and the proportion of cells positive for antibody binding

Analysis of data

Antibody binding can be expressed as

- mean (geometric) in fluorescence channel 1 of:
    - PRBCs (ethidium bromide positive cells) minus that of RBCs (EtBr negative cells), giving the absolute difference in fluorescence, or
    - PRBCs divided by that of RBCs, giving the fold difference in fluorescence levels
- Proportion of PRBCs labelled as positive relative to the mean+3SD of the fluorescence of RBCs

Notes:

- Alternatives to ethidium bromide: SYBR Green I (1:5000) – in this case a different tertiary antibody needs to be used. Eg. Alexa-Fluor 594. In analysis, PRBCs are gated in FL1 and antibody binding is measured in FL3 (for Alexa Fluor 594)
- Fixation: after staining, cells can be fixed with 0.25% glutaraldehyde in PBS for 45 mins
- Incubation volumes: Assay can be succesffuly scaled down to 10ul volumes when using U-bottomed 96-well plates
- Using anti-Ig rather than anti-IgG-specific as the secondary antibody can lead to high background with some isolates
- Using low parasitemias, and no agitation during incubation of cells with antibody, reduces the problem of agglutinates forming
- A 2-step procedure (primary antibody, then labelled secondary antibody) has been used with isolates and serum from children (Kinyanjui SM et al., 2003) and monkey antisera (Baruch DI et al., 2002).

References

Beeson, J. G., Mann, E. M., Elliott, S. R., Lema, V. M., Tadesse, E., Molyneux, M. E., Brown, G. V., and Rogerson, S. J. (2004). Antibodies to variant surface antigens of *Plasmodium falciparum*-

infected erythrocytes and adhesion inhibitory antibodies are associated with placental malaria and have overlapping and distinct targets. J Infect Dis *189*, 540-551.
Piper KP, Roberts DJ, Day K (1999). *Plasmodium falciparum*: analysis of the antibody specificity to the surface of the trophozoite-infected erythrocyte. Exp Parasitol, 91(2):161-9

Staalsoe T, Giha HA, Dodoo D, Theander TG, Hviid L (1999). Detection of antibodies to variant antigens on *Plasmodium falciparum*-infected erythrocytes by flow cytometry. Cytometry, 35(4):329-36.

VIII:G. Analysis of plasma antibodies to variant surface antigens by flow cytometry

by ***Trine Staalsoe***
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Equipment

2-color flow cytometer

Materials and reagents

PBS + 2% FCS (PBS2)
Plasmodium falciparum late-stage infected erythrocytes purified by magnetic separation (see PARASITES, section IV:J) at 2×10^6 erythrocytes/mL in PBS2
ethidium bromide (0.1 mg/mL in PBS)
CAUTION: This chemical is toxic and mutagenic. Wear protective clothing and use only in a chemical fume hood.
human plasma or serum
goat anti-human IgG antibody (DAKO A473)
FITC-conjugated rabbit anti-goat Ig antibody (DAKO F250)

Procedure

Antibody Labeling

- Add ethidium bromide to the erythrocyte suspension (20 µL/mL).
- Mix 100 µL of ethidium bromide-labelled erythrocyte suspension with 5 µL of test plasma/serum and incubate the tube for 30 min at 5 °C.
- Wash the suspension twice in 3 mL of PBS2 and resuspend the sample in 100 µL of PBS2.
- Mix the erythrocyte suspension with anti-human antibody (diluted 1:250) and incubate it as above.
- Wash the suspension twice in 3 mL of PBS2 and resuspend the sample in 100 µL of PBS2.
- Mix the erythrocyte suspension with anti-goat antibody (diluted 1:25), and incubate it as above.
- Wash the suspension once in 3 mL of PBS2 and resuspend the sample in 200 µL of PBS2.
- After antibody Labeling, samples may be kept at 5 °C overnight before analysis.

Data acquisition by flow cytometry

- Adjust flow cytometer settings to achieve a clear separation of uninfected (ethidium bromide-negative) and infected (ethidium bromide-positive) erythrocytes in an FSC/ethidium bromide dot plot (see Fig. 1 in Staalsoe et al. 1999).
- Acquire and store data on FCS, SSC, FL1 (FITC), and FL2 (ethidium bromide).

Data analysis

- Flow cytometry data files (FCS format) can by analysed in any of the many software packages available. We usually use either the freeware WinMDI program (which is good for generating overlays and other graphics for publication) or WinList (which is a very powerful analysis program with wonderful

possibilities for creation of macros that make a breeze of the analysis of the hundreds of files you will often need).

- Set a gate around late-stage infected erythrocytes in an FSC/ethidium bromide diagram plot (similar to R3 in Fig. 1D in Staalsoe et al. 1999).
- Use the FITC fluorescence of the cells within this gate to quantify antibody recognition of variant surface antigens. This can be expressed either as the mean/median fluorescence or as the percentage of cells with FITC fluorescence above that of a control sample incubated without plasma/serum or with control plasma/serum (*viz.*, Fig. 2 in Staalsoe et al. 1999).

Reference

Staalsoe T, Giha HA, Dodoo D, Theander TG, Hviid L. 1999. Detection of antibodies to variant antigens on *Plasmodium falciparum*-infected erythrocytes by flow cytometry. Cytometry 35(4):329-336.

IX. Immunoglobulin- or serum protein-binding to infected erythrocytes

IX:A. Stripping erythrocytes of bound serum proteins and reformation of rosettes

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Equipment

sterile test tubes
centrifuge (500 × *g*)
96-well plates
microscope (400×)

Materials and reagents

500 µL of a rosetting malaria culture (5% hematocrit, rosetting phenotype with a majority of the parsites in trophozoite stage)
PBS (pH 7.2, devoid of Mg^{2+}/Ca^{2+})
heparin, 25000 IU/mL (Leo Pharma)
sodium citrate or PBS-EDTA for stripping

RPMI 1640–HEPES medium (50 mL):
0.52 g RPMI 1640 medium (with glutamine, without $NaHCO_3$; Gibco)
0.3 g HEPES (Gibco)
45 mL distilled water
Mix and set pH to 6.8.

Disruption of rosettes with heparin

Since the rosettes of every parasite strain differ in their heparin-sensitivity, you will have to titrate the necessary heparin-concentration (e.g., FCR3S1.2: 100 U/mL).

- Transfer 500 µL of culture to an appropriate test tube and add the necessary amount of heparin.
- Incubate the culture for 15 min before the next step.

Treatment with sodium citrate or PBS-EDTA to disrupt heparin resistant rosettes

This step can be performed on its own to strip parasitized RBC of bound serum proteins such as immunoglobulins; Both sodium citrate and PBS-EDTA can be used for this purpose. Incubation with 50 mM PBS-EDTA 1 hr at RT is a sufficient alternative protocol. For stripping with sodium citrate follow the protocol below.

- Centrifuge the culture at 500 × *g* and remove the supernatant.
- Add 500 µL of PBS with 100 mM sodium citrate (pH 7.4) to the pellet and incubate it for 5 min.
- Wash the pellet twice with PBS; centrifuge it each time (500 × *g*) and remove the PBS.
- Add 0.25 mL of RPMI 1640–HEPES to the pelleted malaria culture (the hematocrit will be 10%).
  You may include 10% H-Albumin (Sigma), but this can interfere or block cell interactions and the binding to solid surfaces.

Rosette reformation

- Prepare the test substance (e.g., sera or proteins) in 2× concentration of above RPMI 1640 medium.
- Mix 25 μL of the washed culture (10% hematocrit; as above) with 25 μL of the test substance in a 96-well plate. The final hematocrit will be 5% (H-Albumin concentration, if included, will be 5% as well).
- Incubation probably works as well at room temperature as it does at 37 °C for 15 min.
- Read the rosetting rate (=reformed rosettes) using acridine orange (see PARASITES, section III:A)

Reference

Treutiger CJ, Scholander C, Carlson J, McAdam KP, Raynes JG, Falksveden L, Wahlgren M. 1999. Rouleaux-forming serum proteins are involved in the rosetting of *Plasmodium falciparum*-infected erythrocytes. Exp Parasitol 93(4):215-224.

IX:B. Detection of serum proteins on the surface of *Plasmodium*-infected erythrocytes

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Equipment

centrifuge (500 × *g*)
appropriate test tubes for centrifuge
microfuge tubes
glass slides
coverslips
UV-light microscope (100×)

Materials and reagents

malaria cultures (5 to 10% trophozoites)
antibody against serum protein to be detected (e.g., αIgM)
fluorochrome conjugated secondary antibody
RPMI 1640
ethidium bromide (1 µg/mL)
CAUTION: This chemical is toxic and mutagenic. Wear protective clothing and use only in a chemical fume hood.

Immunofluorescence

- The cultures to be tested should have a parasitemia of 5 to 10% with a majority of parasites in trophozoite stages.
- Transfer the culture to appropriate test tubes and wash (500 × *g*) it twice in RPMI 1640. Add RPMI 1640 to the initial volume.
- Dilute antibody to double final concentration of desired dilution in RPMI 1640. (A dilution factor of two will be introduced during the assay because of cell suspension.)
- Aliquot 50 µL of each dilution of antibody in small tubes (e.g., microfuge tubes) and add 50 µL of resuspended culture.
- Mix by shaking the tubes gently and incubate them for 30 min in the dark at room temperature with careful resuspension after 15 min.
- Shake the tube gently after 15 min.
- Wash the cells 3 times (500 × *g*) in 0.5 mL of RPMI 1640.
- Remove the supernatant carefully and add 100 µL of secondary antibody diluted to the final concentration. Mix gently.
- Incubate the cells for 30 min in the dark with careful resuspension after 15 min and wash them as before.
- Resuspend the cells in 40 µL of RPMI 1640 and mix 10 µL of cells with 0.5 to 1 µL of ethidium bromide (1 µg/mL) on a glass slide.
- Add a coverslip and count the fluorescence rate at 100× magnification with oil in a UV-light microscope.

- Calculate the fluorescence rate as = $\frac{\text{number of fluorescent cells}}{\text{total number of late trophozoites}}$

Note: To reduce the amount of background the cells can be incubated with ethidium bromide (1 to 2 µL; 1 µg/mL) for 5 to 10 s before the last wash instead of being mixed with ethidium bromide on the slide.

Note: Count all nonfluorescent cells with the corresponding size of the parasite as the parasites in the fluorescent cells. This is important to achieve the correct fluorescence rate, which could otherwise be too high.

Reference

Scholander C, Treutiger CJ, Hultenby K, Wahlgren M. 1996. Novel fibrillar structure confers adhesive property to malaria-infected erythrocytes. Nat Med 2(2):204-208.

IX:C. Formaldehyde fixation for immunofluorescence analysis (IFA) of *P. falciparum*

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Materials and reagents

snap-top plastic bags
silica gel dessicant
Immunopen
4% formaldehyde (Formaldehyde: Agar Scientific cat # R1026 (10 x 10 mL vials 16% microfiltered solution))
0.1% (v/v) TX100 in PBSA
3% (w/v) BSA in PBSA
PBS with 0.5% (v/v) Tween 20, 1% (w/v) BSA
1 μg/mL 4,6-diamidino-2-phenylindol (DAPI)

Procedure

- Air-dry thin films in a hood for 10-15 min at room temperature, then store in the presence of silica gel dessicant at -70 ^{o}C in snap-top plastic bags.
- For IFA, begin by removing the air-dried thin films from storage at -70 ^{o}C and allow them to reach room temperature still in the snap-top plastic bags containing dessicant. Remove from the bag and draw circles for antibody application with an Immunopen.
- FIXATION: make up 4% formaldehyde fresh by adding one vial (10 mL 16% formaldehyde) to 30 mL PBSA. Place air-dried slides into the fixative and incubate 30 min at room temperature.
- PERMEABILISATION: transfer slides to 0.1% (v/v) TX100 in PBSA at room temperature, incubate 10 min.
- Wash slides twice for 5 min each in PBSA.
- BLOCKING STEP: Block slides for 1 h at room temperature (or overnight at 4 ^{o}C) with 3% (w/v) BSA in PBSA.
- Continue as usual with antibody incubations, diluting antibodies in PBS/0.5% (v/v) Tween 20/1% (w/v) BSA and washing in PBS between steps. Finally counter-stain parasite nuclei with 1 μg/mL DAPI in PBS.

IX:D. Binding and eluting antibodies from the *Plasmodium*-infected RBC surface

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Equipment

immufuge blood centrifuge
round-bottomed test tubes (e.g., 5-mL snap-cap tubes)
microfuge tubes
high speed microcentrifuge with cooling system
high quality pH paper

Materials and reagents

P. falciparum culture with a parasitemia of >8%. (If rosetting is of interest, the rosetting rate should be >50%.)
serum or antibody solution to be investigated
RPMI 1640
PBS (optional)
acridine orange

Solution A:
0.2 M glycine / 0.2 M NaCl:
3.75 g glycine
2.9 g NaCl
Bring to 250 mL in distilled water.

Solution B: (0.2 M HCl)
Mix 1.64 mL of concentrated HCl into 98.4 mL of distilled water.
Note: Mix acid into water, never vice versa with concentrated acids.

Glycine–HCl elution solution (pH 3):
Mix 5 mL of solution A and 1.3 mL of solution B.
Adjust to pH 3 using a high quality pH paper or pH meter.
Make fresh for each day of elution.

Neutralization solution: (2 M Tris-base)
9.69 g Tris-base
Bring to 40 mL; pH will be ~11.

Regarding disruption of rosettes and stripping of serum bound proteins, see Materials and reagents in PARASITES, section IX:A..

If using Percoll/sorbitol gradients to separate trophozoites for binding antibodies to the parasite, see Materials and reagents in PARASITES, section IV:E.

Procedure

- Use at least 1 mL of culture where the parasitemia is >8 to 10%. (If rosetting is of interest, the rate should be >50%; see PARASITES, sections VI:L, M.)

- If purified trophozoites are desired for the antibody binding, use Percoll/sorbitol gradients as in PARASITES, section IV:D. Have a large prep ready with either 12 small bottles (25-cm^2) or 3 large ones (75-cm^2) of culture for 8 Percoll gradients. Follow the protocol for gradients in section IV:D, then resuspend purified cells in 0.5 to 1 mL of RPMI 1640 before the addition of serum/antibodies, followed by the protocol below from the incubation/elution step onward.
- If using the culture straight as it is without trophozoite purification, place the culture in a round-bottomed tube holding a few milliliters and fitting an appropriate blood centrifuge (e.g., a 5-mL snap-cap tube in an immufuge blood centrifuge). Disrupt rosettes using heparin and a syringe, then strip cells of serum-bound proteins (see PARASITES, section IX:A above).
- After the sodium citrate treatment of cells, wash them once with PBS and then with RPMI 1640 (see section IX:A above).
- Add an equal volume of serum or antibody solution to the cell pellet as the starting volume of culture (i.e., mg/mL concentrations of antibody).
- Gently mix and slant the tube so that the blood solution almost reaches the surface of the tube. Carefully roll to mix the settling RBC every 15 min or so. Incubate the tube for 2 to 3 h at room temperature.
- While the tube is incubating, prepare the glycine–HCl elution solution. At the end of the incubation, check that RBC look normal through acridine orange vital staining (see PARASITES, section III:A).
- Spin down the cells (500 $\times$ *g*), remove the supernatant, and wash the cells twice with PBS.
- Elute bound antibodies by adding glycine–HCl elution solution to cells in the amount of the starting culture volume. Incubate them for 1 to 1.5 min only! (It is possible that the bound antibodies can be eluted with less elution buffer for increased antibody concentration; this would have to be tested empirically.)
- Quickly transfer the cells to 2 microfuge tubes and centrifuge them at 8,000 to 10,000 rpm in a chilled centrifuge for 1 min.
- Taking care not to touch the cell pellets, remove the supernatant with the eluted antibodies and pool them into one tube.
- To each milliliter of elution, add 26 to 30 μL of 2 M Tris-base. Mix carefully and use 4 μL to spot on a high quality filter paper. Check the pH. Add more Tris-base, 1 μL at a time, until the pH reaches 7.0 to 7.2. Check the pH after each addition. If the eluate becomes too basic, lower the pH by adding 10 to 20 μL of 0.2 M HCl (= solution B).
- Remove the precipitate that may form by spinning the eluate for a minute in the blood centrifuge.
- If you wish, you may dialyze the eluate overnight against a few changes of PBS in the cold, stirring it slowly.

For storage, you may add sodium azide to 0.02% and a “tiny tad” of pure human serum albumin (e.g., Sigma A3782) if this does not interfere with your subsequent assays.

You can use these eluted antibodies in surface immunofluorescence (see PARASITES, section IX:B above) or in standard Western blotting. Typically, 1:2 or 1:4 dilution of the eluted antibodies (from serum) would be visible in surface immunofluorescence, and 1:10 to 1:20 dilutions in Westerns.

IX:E. Enrichment of immunoglobulin binding parasites using Dynabeads

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Equipment

Magnetic Particle Concentrator (MPC-1, Dynal)
haemocytometer
cryotubes
rotator for incubating cryotubes
25-cm^2 cell culture flask
centrifuge for washing RBC (immunofuge II, Baxter)
microscope (400×)

Materials and reagents

uncoated Dynabeads M-450 Tosylactivated (90 mg/mL, Dynal)
appropriate antibodies for coating the Dynabeads
0.1 M borate
PBS 0.1% HSA (50 mL PBS + 50 mg albumine, filter-sterilized)
PBS 10% HSA (50 mL PBS + 5 g albumine, filter-sterilized)
acridine orange
RPMI 1640
MCM without serum (see PARASITES, section I:A)
MCM with 10% serum (see PARASITES, section I:A)

Enrichment of the parasites

- Coat uncoated Dynabeads M-450 Tosylactivated with antibodies using the protocol provided by Dynal.
- Count the parasitemia of the *Plasmodium falciparum* culture and determine the stages of the parasites using acridine orange (see PARASITES, section III:A). It should have a parasitemia of approximately 10% and the parasites should be in trophozoite stages.
- Determine the number of erythrocytes per milliliter using a haemocytometer. Calculate the number of target cells (=infected RBC) by multiplying the number of cells with the parasitemia. One or two milliliters of parasite culture (approximately 5×10^7 target cells) is usually enough to achieve a good parasitemia after enrichment.
- Calculate the volume of Dynabeads to have 6 Dynabeads per target cell in the solution.
- Wash the Dynabeads 3 times with 3 mL of RPMI 1640 using a 3.6-mL cryotube and a Magnetic Particle Concentrator.
- Wash the culture 3 times (500 × *g*) with MCM without serum (see PARASITES, section I:A).
- Resuspend the erythrocytes in approximately 2 mL of RPMI 1640 and add the RBC to the Dynabeads in the cryotube.
- Immediately incubate the tube on a rotator at room temperature for 1.5 to 2.0 h, during which time the trophozoites will attach to the coated Dynabeads.

- After the incubation, before the beads/erythrocytes have time to settle, use the Magnetic Particle Concentrator to remove the medium while the PRBC attached to magnetic beads are bound to the MPC-1.
- Wash the beads/erythrocytes very gently, 1 or 2 times with RPMI 1640 using the Magnetic Particle Concentrator.
- Resuspend the beads/erythrocytes in MCM with 10% serum (see PARASITES, section I:A) in a 25-cm^2 cell culture flask at a hematocrit of 5% (i.e., 3.8 mL of MCM with serum, 0.2 mL of packed erythrocytes, and the Dynabead-bound *Plasmodium falciparum* infected erythrocytes in a 25-cm^2 flask).

During the next day, remove the beads from the culture using the Magnetic Particle Concentrator. The parasitized erythrocytes should by then have infected the free erythrocytes in the culture. Continue culturing the remaining erythrocyte mixture as described in PARASITES, section I.

Reference

Heddini A, Treutiger CJ, Wahlgren M. 1998. Enrichment of immunoglobulin binding *Plasmodium falciparum*-infected erythrocytes using anti-immunoglobulin-coated magnetic beads. Am J Trop Med Hyg 59(5):663-666.

X. Fractionation of the iRBC

X:A. *Plasmodium-infected* erythrocytes: separation of ghosts from parasite membranes

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Equipment

centrifuge (10.000 × g), refrigerated
appropriate reaction tubes for centrifuge

Materials and reagents

Buffer A (hypotonic lysis buffer): 5 mM KH_2PO_4, pH 7.4
Buffer B (washing buffer): 100 mM PBS, pH 7.4
buffers contain:
Protease Inhibitor Cocktail Set III (Calbiochem), dilution 1:500

Procedure

- Use the pellet of $1x10^8$ – $5x10^8$ enriched trophozoite stage parasites (~ 80% parasitemia).
- Resuspend the pellet in 0.4 mL buffer A and incubate for 10 min at RT (vortex every 2 min).
- Centrifuge the sample for 10 min at 10.000 x g in a microfuge (4 °C).
- To avoid cross contamination separate about 80% of the opaque supernatant (ghost fraction).
- Discard the remaining ghosts as well as 20% of the remaining parasites containing pellet.
- Wash the parasite pellet twice with 0.4 mL of buffer B (store at -20 °C).
- Spin down the ghost fraction at 10.000 x g for 20 min at 4 °C and wash the sediment with 0.5 mL buffer B (store at -20°C).

Comments

if available use 400 µL test tubes instead of 1.5 mL tubes. Because of the smaller diameter the ghost layer is bigger and separation much easier.

1.5 0.4 mL reaction tube

X:B. Subcellular fractionation of iRBC: use of saponin and streptolysin O

*by **Stefan Baumeister**, **Markus Winterberg** and **Klaus Lingelbach***
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General comments

Treatment of infected erythrocytes with Streptolysin O (SLO) results in the release of the erythrocyte cytosol. Parasites contained within an intact parasitophorous vacuole can be sedimented by centrifugation. Treatment of infected erythrocytes with saponin results in the disintegration of the erythrocyte membrane and the parasitophorous vacuolar membrane. Intact parasites can be sedimented by centrifugation.

Permeabilization of iRBC with Streptolysin O

Equipment

centrifuge, refrigerated
counting chamber

Materials and reagents

Buffers:
PBS^{++}: 10 mM NaH_2PO_4, 10 mM Na_2HPO_4, 145 mM NaCl, 3 mM KCl, pH 7,2
buffer contains: Protease Inhibitor Cocktail Set III (Calbiochem), dilution 1:500
Streptolysin O (SLO): kindly provided by S. Bhakdi , Mainz, Germany
100 µg of lyophilized protein are dissolved in 1,12 mL PBS^{++} and stored on ice for determination of hemolytic units (see below)

Alternative: Preparation of SLO from SIGMA see below

Procedure

- Incubate IRBC (in aliquots of 2 x 10^8 cells) in 200 µL PBS with 3–4 hemolytic units of SLO at room temperature for 6 min.
- Centrifuge samples at 10,000 x g for 15 s.
- Save supernatant (containing the cytosol of the infected RBC).
- Wash the pellet (containing intact parasites, the vacuolar contents, and membranes) twice with 200 µL of PBS.
- Fractions are now ready for further analyses.

Determination of hemolytic units (HU)

1 hemolytic unit is determined as the amount of dissolved SLO necessary for the lysis of 50 % of red cells

- Take ~200 µL of packed red blood cells and wash three times with PBS.
- Resuspend in 1 mL PBS^{++}.
- Count cells with counting chamber.
- Aliquot 10^8 cells in 6 cups (I – VI).
- Cup I: **hemoglobin control**, adjust cell suspension to final volume of 100 µl, lyse cells by three freeze/thaw-circles, spin at 10,000 x g, 20 min, 4 °C and dilute supernatant 1 : 500.
- Extinction at 412 nm gives total amount of haemoglobin.

- Cups (II – VI): **SLO-lysis,** adjust cell suspension to 90µL and suspend cells thoroughly, add to each cup increasing amounts of SLO (6, 8, 10, 12, 14 µL) and gently vortex each cup, incubate for 6 minutes at room temperature, spin at 10,000 x g, 20 sec, 4 °C and dilute supernatants 1 : 500.
- Extinction at 412 nm gives the released amount of haemoglobin.
- Choose amount of SLO sufficient for hemoglobin release of ~100 % (2 HU).
- Make proper aliquots of SLO-solution and store at -80°C.

Use of Sigma SLO

Sigma provides SLO as a lyophilized powder with a total activity of 25,000 units

Buffers: 10 mM PBS pH 7.4, 100 mM NaCl
1 M DTT (in water or PBS)

- Dissolve SLO in 2.25 mL PBS.
- Make aliquots of 90 µL and freeze them at -20° C.
- Thaw 90 µl SLO and add 10 µl of 1 M DTT (in water or PBS) - incubating 15 min at RT yields activated SLO (SLO*).
- Use 300 units SLO* for 1 x 10^8 iRBC:
- 10^8 iRBC are dissolved in 70 µL PBS
- Add 30 µL SLO*.
- Incubate 10 min at RT.

Permeabilization of iRBC with Saponin

Materials and reagents

Buffers:
PBS^{++}: 10 mM NaH_2PO_4, 10 mM Na_2HPO_4, 145 mM NaCl, 3 mM KCl, pH 7,2
buffer contains: Protease Inhibitor Cocktail Set III (Calbiochem), dilution 1:500

Saponin from SIGMA

Procedure:

- for saponin lysis, incubate iRBC (in aliquots of 2 x 10^8 cells) in 200 µL of 0.1% saponin in PBS pH 7.2 on ice for 5 min.
- centrifuge the samples at 2,500 x g for 5 min
- remove supernatant (containing host cytosol and vacuolar contents)
- wash the pellet (containing the intact parasite) twice with 200 mL of PBS before further processing

References

Ansorge I., Benting J., Bhakdi S., Lingelbach K. (1996) Protein sorting in *Plasmodium falciparum*-infected red blood cells permeabilized with the pore-forming protein streptolysin O. Biochem J 315: 307–14.
Baumeister, S., A. Burgwedel, U.G. Maier and Lingelbach, K. (1999) Reconstitution of protein transport across the vacuolar membrane in *Plasmodium falciparum*-infected permeabilized erythrocytes. Novartis Found Symp 226: 145–154.

Burghaus, P.A. and Lingelbach, K. (2001) Luciferase, when fused to an N-terminal signal peptide, is secreted from transfected *Plasmodium falciparum* and transported to the cytosol of infected erythrocytes. J Biol Chem 276 : 26838–26845.
Nyalwidhe, J., Baumeister, S., Hibbs, A.R., Tawill, S., Papakrivos, J., Volker, U., and Lingelbach, K. (2002) A nonpermeant biotin derivative gains access to the parasitophorous vacuole in *Plasmodium falciparum* infected erythrocytes permeabilized with streptolysin O. J Biol Chem 277: 40005–40011.
Nyalwidhe, J. and Lingelbach, K. (2006) Proteases and chaperones are the most abundant proteins in the parasitophorous vacuole of *Plasmodium falciparum*-infected erythrocytes. Proteomics 6: 1563–1573

X:C. Purification of cholesterol-rich membrane microdomains (DRM-rafts) from *Plasmodium* infected erythrocytes

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Equipment

Eppendorf microfuge
Beckman ultracentrifuge
SW60 rotor
Dounce homogenizer

Materials and reagents

Purified infected RBC (2-5x10^8)
Erythrocyte lysis buffer (10x stock solution):
- 1.5M NH_4Cl
- 0.1M $KHCO_3$
- 0.01M EDTA

Parasite lysis buffer:
- 25mM MES pH 6.5
- 0.15 M NaCl
- 1% TritonX-100

Protease inhibitors (Roche tablets)
MES buffered saline:
- 25mM MES pH6.5
- 0.15M NaCl

80% sucrose in MES buffered saline (solution A)
30% sucrose in MES buffered saline (solution B)
5% sucrose in MES buffered saline (solution C)

Procedure

Erythrocyte lysis:

- Resuspend purified infected RBC pellet in 2 mLof cold 1x erythrocyte lysis buffer.
- Incubate this suspension in ice until lysis is completed (10-15 min) and spin at 4°C in 1.5 mLEppendorf tubes (5 min at 6000 rpm) to collect free parasites.
- Wash the pellet several times at the same speed with cold PBS to remove soluble hemoglobin.
- *Comment:* this procedure can be adopted to lyse both human and rodent infected RBC. Hemoglobin removal is less efficient than that obtained by saponin treatment; however saponin can't be used because it partially removes cholesterol from membranes thus affecting lipid rafts integrity.

DRM-rafts purification:

- Add 720 μL of cold parasite lysis buffer to the pellet kept on ice (2-5x10^8 parasites) and homogenize cell extract with 10 strokes of a Dounce homogenizer.
- Transfer cell lysate in an ultracentrifuge tube (Beckman, 11 x 60 mm) and adjust to 40% sucrose solution by adding 750 μL of solution A.
- Throughly overlay with 1.5 mLof solution B and 1.5 mLof solution C.

- Centrifuge at 45,000 rpm for 16-20 h at 4°C in a SW 60 rotor (Beckman Instruments).
- Collect 370 μL fractions from the top of the gradient (12 fractions). DRM-rafts appear as anopaque band migrating at 10-20% sucrose (fractions 4 and 5).

Comments

It is important that the entire procedure is performed in the cold to avoid solubilisation of raft-associated proteins during cell extract preparation

References

Sargiacomo, M., Sudol, M., Tang, Z., and Lisanti, M. P. (1993) Signal transducing molecules and glycosyl-phosphatidylinositol-linked proteins form a caveolin-rich insoluble complex in MDCK cells. *J. Cell Biol.* 122, 789-807

IV:D. Obtaining free parasites

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SEE: IV.G. page 38

XI. In vitro reinvasion and growth inhibition assays

XI:A. In vitro reinvasion and growth inhibition assay by microscopy of erythrocyte monolayers

Method developed by ***Birgitta Wahlin-Flyg***

Equipment

centrifuge
UV-microscope

Materials and reagents

human type O+ RBC
parasitized red blood cells (PRBC)
TCM:
HEPES-buffered (20 mM) RPMI 1640 medium
10% normal human serum
2 mM glutamine
gentamycin (25 μg/mL)
0.2% $NaHCO_3$
Tris-buffered Hanks' solution (TH)
0.06 M bicarbonate buffer (pH 9.6)
1% glutaraldehyde in PBS
acridine orange (10 μg/mL)
96-well flat-bottomed tissue culture plates
conical tubes
8-well multitest slides
coverslips

Procedure

- Using normal human type O^+ RBC, dilute parasitized red blood cells (PRBC) from *Plasmodium falciparum* cultures infected with late trophozoites and schizonts to a parasitemia of approximately 1% and adjust the hematocrit to 2% with TCM.
- Set up quadruplicate tests in 96-well flat-bottomed tissue culture plates. Use 100 μL of parasite culture mixed with 100 μL of TCM or various dilutions of antibodies.
- Incubate the plate at 37 °C for 18 to 20 h in a candle jar (see PARASITES, section I:A).
- Transfer the quadruplicate tests separately to 4 conical tubes, wash them by centrifugation 2 times with 1 mL of Tris-buffered Hanks' solution (TH), followed by a dilution to 1% hematocrit, by adding 150 μL of TH.
- Make monolayers in duplicates from each tube on 8-well multitest slides that have been pretreated for 30 min with 0.06 M bicarbonate buffer (pH 9.6).
- After 30 min, wash the slides in TH, fix them by two treatments for 10 s each with 1% glutaraldehyde in PBS, wash them with distilled water, and air-dry them.
- Store the slides at room temperature until they are analysed in the UV-microscope.

Analysis of percent parasitemia by UV-microscopy

- To analyse the parasites, stain by adding one drop of acridine orange (10 μg/mL) per well for a few seconds, then wash the slides with distilled water and mount a coverslip.
- Screen 25 microscope fields per well (200 visual fields/slide). Since the number of RBC/microscope field has been estimated to be 200/field, the percent parasitemia can be calculated from a total of 40,000 RBC/culture:
- Calculate the percent parasitemia as:

$$\frac{(\text{percent parasitemia in control} - \text{percent parasitemia in sample}) \times 100}{\text{per cent parasitemia in control}}$$

Reference

Udomsangpetch R, Wåhlin B, Carlson J, Berzins K, Torii M, Aikawa M, Perlmann P, Wahlgren M. 1989. *Plasmodium falciparum*-infected erythrocytes form spontaneous erythrocyte rosettes. J Exp Med 169(5):1835-1840.

XI:B. In vitro reinvasion and growth inhibition assay by flow cytometric measurement of parasitemia using propidium iodide (PI) staining

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This method allows for quantification of parasitemia and for quantification of parasites in different stages of differentiation according to DNA content. Many similar protocols have been published using different intercalating dyes such as acridine orange, thiazole orange, hydroethidine, or YOYO-1. Each of them has advantages and disadvantages. Below we describe our variant that we find easy and reliable. Care should be taken to distinguish nucleated cells from parasitized erythrocytes when analyzing material containing nucleated cells. The protocol can be extended by additional labeling of cells using FITC-, PE-, and APC-labeled antibodies.

Equipment

centrifuge
flow cytometer
FACScan (Becton-Dickinson)
FACScalibur (Becton-Dickinson)

Materials and reagents

PBS
PBS containing 0.025% (v/v) glutaraldehyde
PBS containing 0.01% saponin
PBS containing 2% FCS
propidium iodide (PI)

Procedure

- Take 1×10^6 to 2×10^6 red blood cells (RBC) from a culture or a drop of blood from an infected individual collected into heparinized isotonic buffer (e.g., PBS or 0.9% sodium chloride).
- Wash the cells in PBS twice.
- To fix the cells, resuspend them in 1 mL of PBS containing 0.025% (v/v) glutaraldehyde, and incubate them at room temperature for 20 min. Alternatively, incubate the cells at 4 °C for 30 to 45 min. Cells may be stored in the fixative for several weeks before proceeding.
- Centrifuge the cells for 5 min at 450 × *g*. Aspirate the supernatant followed by two washes in PBS.
- Permeabilization:
  - Resuspend the cells in 0.5 mL of PBS containing 0.01% saponin.
  - Incubate them at room temperature for 5 min.
- Wash the cells in PBS twice at 450 × *g* for 5 min.
- Resuspend the cells in 0.5 to 1.0 mL of FACS buffer. (We use PBS containing 2% FCS.).
- Staining with propidium iodide: To the above cell suspension, add PI to a final concentration of 10 μg/mL. Incubate the cells at 37 °C for 1 to 2 h.
- Analyze the cells in a flow cytometer with a 488-nm laser for excitation, and detect emission from intercalating PI in the 670-nm long pass filter (red FL3

channel). We use the FACScan and the FACScalibur from BD and detect emission in FL3.

References

Barkan D, Ginsburg H, Golenser J. 2000. Optimisation of flow cytometric measurement of parasitemia in *Plasmodium*-infected mice. Int J Parasitol 30(5):649-653.
Makler MT, Lee LG, Recktenwald D. 1987. Thiazole orange: a new dye for *Plasmodium* species analysis. Cytometry 8(6):568-570.
Pattanapanyasat K, Udomsangpetch R, Webster HK. 1993. Two-color flow cytometric analysis of intraerythrocytic malaria parasite DNA and surface membrane-associated antigen in erythrocytes infected with *Plasmodium falciparum*. Cytometry 14(4):449-454.
Whaun JM, Rittershaus C, Ip SH. 1983. Rapid identification and detection of parasitised human red cells by automated flow cytometry. Cytometry 4(2):117-122.
Wyatt CR, Goff W, Davis WC. 1991. A flow cytometric method for assessing viability of intraerythrocytic hemoparasites. J Immunol Methods 140(1):23-30.

XI:C. *Erythrocyte invasion assay*

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see: Transfection, page 382

XI:D. *P. falciparum* Growth or Invasion Inhibition Assays using Antibodies

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Materials and reagents

RPMI-HEPES with 5% pooled human serum
Albumax II
glutamine
hypoxanthine
gentamicin
U-bottom 96 well tissue culture plate (Falcon 3077)
CPD buffer

Procedure

Growth inhibition assays performed over one or two cycles of parasite replication

- Repeatedly synchronise parasite cultures in the 1-2 weeks prior to performing the assay. Recommended methods for synchronisation:

1. Sterile 5% sorbitol (in water): performed when parasites are mainly ring forms by resuspending parasite culture pellet in sorbitol solution, incubating for 5 mins, centrifuge at 1500 rpm for 5 mins, then resuspend pellet back into culture medium
2. Gelatin enrichment of pigmented trophozoites: involves floatation of mature-stage parasites in 0.75% gelatin
3. Magnet-purification of mature pigmented parasitized RBCs using a MACS column

- Use highly synchronous parasite culture in the assays (this is critical). Most PRBCs must be at the late pigmented trophozoite or early schizont stage. Do not use if cultures have many ring-stage parasites

Setting up the assay:

- Starting parasitemia should be around 0.5 – 0.8% for a one-cycle assay, and around 0.1-0.3% for a two-cycle assay. Reduce the parasitaemia of starting culture by adding fresh RBCs as needed.
- Prepare parasites: resuspend parasites at 1% haematocrit (eg 50ul of cell pellet in 5ml of medium) in RPMI-HEPES with 5% pooled serum and 0.25% Albumax II (0.25% equals 2.5g in 1 litre). RPMI-HEPES must be properly supplemented with glutamine (2mM) and hypoxanthine (50μg/ml). Good to also include gentamicin to help prevent bacterial contamination.
- Setting up the plates: Use U-bottom 96 well tissue culture plate (Eg. Falcon 3077). Test all samples in duplicate. Include positive and negative controls in every assay run (Eg. inhibitory antibodies, samples from non-exposed donors). Include negative controls on every plate if setting up multiple plates (can use method A or B).

Method A:
- Aliquot samples to be tested in inhibition assays into U-bottom 96 well plates. Keep plates on ice to avoid samples evaporating
- Add parasite suspension to each well of the 96 well culture plate. Final volume should be 25 or 50 μl. For consistency, use a multi-pipettor (Eg. Eppendorf) or multi-channel pipettor to add parasite suspension to wells. Cover wells with plate lid.

Method B:
- Add parasite suspension (as above) to plates first
- Add samples to each well

- Prepare a smear from the remaining parasite sample used for the assay – use this smear later to determine the parasitemia and stage of culture (then store these smears for later reference)

- Put plate into humidified chamber, gas (with mix of 95% nitrogen, 4% CO_2, 1%O_2), seal chamber and incubate at 37°C. For incubation chamber, best results are obtained with specialized culture chambers. Include wet paper towels or tissues inside the chamber to create a humid environment. Be sure to sit the test plates on a blank or empty plate to avoid direct contact of the test plates with wet paper towels (to avoid possible contamination of cultures with yeast or bacteria). Be sure to gas the incubation chamber fully. It may be preferable to repeat this after 15 minutes.

- For two cycle assays: at 48 hours, add fresh medium to each well (do not remove existing medium). For 25 μl cultures, add 5 μl. For 50μl cultures, add 10μl.

- Agitation: Twice daily gentle agitation for 1 minute (or until pellet is resuspended) of the culture chamber may help redistribute parasites and create more even growth in the wells. Our results suggest this slightly increases the sensitivity of the assay, but it is not essential.

- Keep a sample culture dish or flask in incubator to monitor developmental stage of parasites to guide the timing of harvesting parasites

- Measure parasitemia (preferably by flow cytometry, but can also be done by microscopy or pLDH-based assays)

    - One-cycle assay: measure parasitemia at 24-48 hours (or microscopy at 24 hours) by flow cytometry. Best to measure parasitemia when parasites are at late ring or early trophozoite stage if measuring parasitemia by flow cytometry.

    - Two-cycle assay: measure parasitemia at 80-96 hours (this varies with the parasite line used)

Comments

Culture volumes in 96-well plates can be 25-100 μl. We routinely use 50 μl volumes. Growth rates at 50μl are generally a little higher than 25 μl cultures. U-bottom plates are more practical, and growth rates are slightly better for U bottom plates

Avoid using culture medium prepared with Albumax only (without serum)

Use only sterile equipment, aliquot samples and parasites in class II hood

Always include non-immune controls (and positive controls if possible)

Clean the glass or plastic incubation chamber before and after use to avoid the build-up of yeast that may contaminate cultures.

Always use fresh RBCs in the starting culture. Store RBCs in CPD buffer, not PBS or RPMI-HEPES

Notes on testing human serum or plasma samples:

- Test samples at 1/10 dilution or less. Testing samples at a 1/5 dilution may lead to problems with non-specific inhibition
- Ideally, all samples should be dialysed to equilibrate pH and remove non-specific inhibitors (Eg. antimalarial drugs and antibiotics), or purification of immunoglobulins performed
- Avoid repeated freeze-thaw cycles with serum/plasma samples
- Purification of immunoglobulins can be performed with ammonium sulfate precipitation (Persson et al 2006), protein G columns, or using Melon Gel (Thermo/Pierce), followed by dialysis against PBS

Measuring parasitemia by flow cytometry:

- Best performed when parasites are at the late-ring to mid-pigmented trophozoite stage
- Prepare PBS with ethidium bromide at 10 μg/ml (use molecular biology grade ethidium bromide). Alternatively, hydroethidine or Sybr Green (1:5000) can be used
- Add 100μl of PBS-EtBr to each well of the 96-well plate
- Incubate in darkness for 30-60 mins
- Centrifuge plate (1200 rpm, 1 minute) to pellet cells, and remove supernatant
- Resuspend parasites into 200-300ul of PBS and transfer samples to FACS tubes (cover samples from exposure to light). When using a high throughput sampler with the flow cytometer, samples can be taken directly from the 96well plate rather than transferring to FACS tubes
- Measure parasitemia on flow cytometer (Ethidium bromide is detected in channel Fl2)

References

Persson, K. E. M., Lee, C. T., Marsh, K., and Beeson, J. G. (2006). The development and optimization of high throughput methods to measure *Plasmodium falciparum*-specific growth inhibitory antibodies. J. Clin. Microbiol. *44*, 1665-1673.

McCallum, F.J., Persson, K.E.M., Mugyenyi, C.K., Fowkes, F.J., Simpson, J.A., Richards, J.S., Williams, T.N., Marsh, K., and Beeson, J.G. (2008). Acquisition of growth-inhibitory antibodies against blood-stage *Plasmodium falciparum*. PLoS ONE 3, e3571.

Persson, K.E.M., McCallum, F.J., Reling, L., Lister, N.A., Stubbs, J., Cowman, A.F., Marsh, K., and Beeson, J.G. (2008). Variation in use of erythrocyte invasion pathways by *Plasmodium falciparum* mediates evasion of human inhibitory antibodies. J Clin Invest *118*, 342-351.

XI:E. ^{3}H-hypoxanthine incorporation assay for the study of growth inhibition by drugs

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Apparatus

speed vac (Savant Plus SC 110 A)
Harvester (Tomtec Mach III)
scintillation counter (1450 Microbeta Liquid)
Candle jar
Bag sealer

Equipment

96-well cell culture plates, flat-bottomed with lid (Costar)
Eppendorf tubes
Falcon tubes, 14-mL
pipettes, tips 5- to 200-µL
dispenser (Eppendorf Multipette plus)
Eppendorf Combitip Plus for 0.5 mL
filter papers (Printed Filtermat A Wallac)
sample bags (Wallac)
gloves
racks

Materials and reagents

parasite strain (FCR 3 S1) in culture (unsynchronized)
complete culture medium: RPMI 1640 with 10% human serum from blood group AB+ = MCM (see PARASITES, section I:A)
human erythrocytes, blood type O^{+}
test compounds dissolved in DMSO (in conc. 5 mM)
DMSO (dimethyl sulfoxide)
acridine orange (see PARASITES, section III:A)
Beta-plate scintillation fluid
chloroquine diphosphate, in two concentrations, 40 nM and 640 nM, as a control drug

stock solution for control drug 1 (64×10^{-5} M chloroquine diphosphate (MW: 515.9 g/mol):

33 mg chloroquine diphosphate
100 mL distilled water.

Prepare the stock solution. Store at 4 °C in the dark in a plastic flask. (The drug adheres to glass.)

working solution for control drug 1:

Prepare the working solutions of control drug 1 by diluting the stock solution by 1:100 in RPMI 1640 (containing 1% DMSO); 64×10^{-7} M = control drug 1

working solution for control drug 2:

Make two-fold dilutions of the working solution for control drug 1 in 4 steps; 4×10^{-7} M = control drug 2

8-^{3}H-hypoxanthine (Amersham), 1 mCi/mL in 50% ethanol
Aliquot 100 μL (in 100-μL Eppendorf tubes) and store at −20 °C.
Prepare the working solution by evaporating the alcohol in a speed vac.
Resuspend the isotope in 5 mL of RPMI 1640 (i.e., 20 μL isotope/mL of RPMI 1640).
Prepare a new working solution for each assay.

Preparation

Day 1

Work in a sterile hood and use sterile tips and tubes.

- Dilute the test compounds (25 per microplate)
  A: 1/1005 μL in 0.5 mL RPMI
  B: 1/100050 μL of A in 0.5 mL RPMI
  Final concentrations will be: A: 5 μM, B: 0.5 μM

- Prepare a dilution of washed, uninfected human type O^+ erythrocytes at 2% hematocrit in MCM as background.
  Example: 20 μL O^+ blood in 1 mL MCM. Add 1 μL DMSO (to achieve the same concentration as in the test samples).

- Estimate the parasitemia (*X*%) in the malaria culture and prepare a parasite suspension with 0.2% parasitemia and 2% hematocrit. A 20-mL suspension is enough for 3 plates. (Calculate the amount of parasite culture to add for a 0.2% parasitemia in 20 mL of MCM, add 0.4 mL of O^+ blood for 2% hematocrit.)

	B 2	**B 3**	2 A	2 B	8 A	8 B	14A	14B	20A	20B	
	C 2	**C 3**	3 A	3 B	9 A	9 B	15A	15B	21A	21B	
	D 2	**D 3**	4 A	4 B	10A	10B	16A	16B	22A	22B	
	E 2	**E 3**	5 A	5 B	11A	11B	17A	17B	23A	23B	
	F 2	**F 3**	6 A	6 B	12A	12B	18A	18B	24A	24B	
	1 A	1 B	7 A	7 B	13A	13B	19A	19B	25A	25B	

- Fill all wells around the border of the 96-well plate with a few drops of RPMI.
- Add 10 μL of control drug I to each of the wells E2 and E3.
- Add 10 μL of control drug II to each of the wells F2 and F3.
- Add 10 μL of the test compounds in dilutions A and B above to each of the wells 1A, 1B (G2, G3) to 25 A, 25B (G10, G11), resulting in 25 compounds in two dilutions per plate.
- Add 90 μL of the uninfected erythrocytes to each of the wells B2 and B3 for background.
- Add 90 μL of the parasite suspension to each of the wells C2, C3, D2, and D3 for controls.
- Add 90 μL of the parasite suspension to each of the remaining wells with control drugs and test compounds.

- Continue in the same way with the next plate.
- Note the date, starting time, and number of the plate on the lids.
- Put the plates in a candle jar and incubate them at 37 ^{o}C for 24 h. Use a thin layer of sterile water on the bottom of the jar.

Day 2

- Add 25 μL of the ^{3}H-hypoxanthine working solution to each well (0.5 μL/well) and continue the incubation for another 18 h.

Day 3

- Harvest the cells to labeled filter papers in a cell harvester and dry them in an incubator for 1 h.
- Put the filters in sample bags and add 3.2 mL of scintillation fluid.
- Seal the bags and see that the fluid is evenly distributed over the filters.
- Read the cpm in a MicroBeta counter.
- Estimate the inhibition:

$$\% \text{ inhibition} = 100 - \frac{[(\text{cpm in test well} - \text{cpm in B2,3}) \times 100]}{\text{cpm in C2,3 D2,3} - \text{cpm in B2,3}}$$

Control drug 1 inhibits the growth of FCR3 S1 by >90%
Control drug 2 inhibits the growth of FCR3 S1 by 10 to 30%

References

Chulay JD, Haynes JD, Diggs CL.1983. *Plasmodium falciparum*: Assessment of in vitro growth by ^{3}H-hypoxanthine incorporation. Exp Parasitol 55(1):138-146.

Desjardins RE, Canfield CJ, Haynes JD, Chulay JD. 1979. Quantitative assessment of antimalarial activity in vitro by a semiautomated microdilution technique. Antimicrob Agents Chemother 16(6):710-718.

XI:F. *Plasmodium falciparum* hemozoin formation assay

by ***Mats Wahlgren***
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Equipment

high speed Immufuge blood centrifuge
centrifuge with Beckman JA-20 rotor
vortex
spectrophotometer (560-nm)

Materials and reagents

malaria culture
4-mL Falcon tubes (BD Labware, 2058)
1.5-mL microcentrifuge tube
5% sorbitol, prepared in distilled water and filtered through a 0.22-μm filter
MCM with 10% serum
Triton X-100
distilled water
N NaOH
pyridine
2.5 mM potassium ferricyanide, $K_3Fe(CN)_6$
sodium hydrosulfite, $Na_2O_4S_2$
ferriprotoporphyrin IX chloride (Fluka or Sigma) (optional)

Procedure

Grow *Plasmodium falciparum* in MCM medium containing 10% human type AB^+ serum and 5% hematocrit (type O^+ blood) according to the candle-jar method of Trager and Jensen (see PARASITES, section I:A).

Synchronization of parasites (see also PARASITES, sections IV)

- Perform 1 cycle (or more if necessary) of synchronization by sorbitol lysis. Choose a culture with a majority of rings.
- Spin the 4-mL culture for 1 min at “high” speed in an immufuge blood centrifuge using 4-mL Falcon tubes.
- Add 4 mL of 5% sorbitol to the pellet.
- Mix the culture thoroughly by inversion and let it stand for 10 min.
- Spin again for 1 min.
- Wash the pellet 3 times in MCM with 10% serum.
- Return the cells to a culture flask.
- Wait until next ring stage period. Repeat the synchronization 2 more times if necessary.

Growth of parasites with drugs

- Use cultures at the ring stage (12 to18 h) and a parasitemia of ~5%.
- Change the cultures to fresh MCM (with 10% serum) before adding the drugs.
- Add test drugs to a final concentration of 1 or 10 μg/mL (or concentrations in between).

- Controls:
  - untreated PRBC culture (as background)
  - PRBC culture treated with chloroquine, or other anti-malarial drugs (e.g., Fansidar), at 1 and 10 μg/mL
- Mix by inversion and split the culture into flasks or wells in a cell culture plate.
- Grow a 4-mL culture for each time point in a 25-cm^2 cell culture flask or a 2.5-mL culture for each time point in a 6-well plate.
- Allow the culture to mature for ~30 h with or without an inhibitory drug.

Pyridine-hemochrome method for the measurement of haem incorporation in hemozoin

- Harvest the culture at various time points spread over ~30 h and measure hemozoin content; e.g., use cultures at: 0 h, 18 h, 20 h, 25 h, and 30 h.
- Transfer contents of the flasks/wells to 10-mL centrifuge tubes which hold for the centrifugation below.
- Add Triton X-100 to a final concentration of 1% (4 mL of a 2% Triton X-100 solution to a 4-mL culture). Leave a small volume of culture for 5% Giemsa staining (see also PARASITES, section III:B) and for counting the parasitemia.
- Spin at 4 °C and 13,000 rpm for 45 min (Beckman JA-20 rotor).
- Discard the supernatant and save the pellet.
- Resuspend the pellet with 1 mL of distilled water and transfer it to a 1.5-mL microcentrifuge tube.
- Spin it at 4 °C and 13,000 rpm for 15 min in a microcentrifuge. This step can be repeated to further wash away any free haem.
- Discard the supernatant and save the pellet.

Do the following steps in a VENTILATED HOOD since pyridine and potassium ferricyanide are VERY TOXIC. Collect waste in a flask, label it appropriately, and send it as organic waste.

- If one wishes to set up a standard curve for the assay, one can use hemin which is ferriprotoporphyrin IX chloride (Fluka or Sigma). Make standard working solutions in distilled water from a stock solution of hemin prepared in DMSO, as hemin is not directly soluble in water. Then take 520 μL of these prepared hemin standard solutions and treat them exactly as the dissolved hemozoin pellet below.
- Add 520 μL of distilled water to the tube with the culture hemozoin pellet, followed by 62 μL of N NaOH and 123 μL of pyridine (for a 4-mL sample). Use half of these volumes for a 2.5-mL sample. Vortex to dissolve the pellet. Add the same volumes of NaOH and pyridine to the hemin standard tubes.
- Split the mixture into equal parts in 2 microcentrifuge tubes. Add 20 μL (or 10 μL for a 2.5-mL sample) of 2.5 mM potassium ferricyanide to one tube to oxidize haem. Add “a pinch” of sodium hydrosulfite to the other tube to reduce haem. Mix by inversion.
- Measure the absorbance in a spectrophotometer at the wavelength of 560 nm.
- Calculate the relative amount of hemozoin at each time point as well as for the hemin standards:
  - Δ OD_{560} = OD_{560} (reduced sample) – OD_{560} (oxidized sample)

- Plot hemozoin content in the culture versus time and compare relative to culture without drugs and to culture with chloroquine and/or Fansidar, as well as to the standards.

References

Asawamahasakda W, Ittarat I, Chang C-C, McElroy P, Meshnick SR. 1994. Effects of antimalarials and protease inhibitors on plasmodial hemozoin production. Mol Biochem Parasitol 67(2):183-191.

Francis SE, Gluzman IY, Oksman A, Knickerbocker A, Mueller R, Bryant ML, Sherman DR, Russell DG, Goldberg DE. 1994. Molecular characterization and inhibition of a *Plasmodium falciparum* aspartic hemoglobinase. EMBO J 13(2):306-317.

Slater AFG, Cerami A. 1992. Inhibition by chloroquine of a novel haem polymerase enzyme activity in malaria trophozoites. Nature 355(6356):167-169

XI:G. Erythrocyte invasion assays with isolated viable *P. falciparum* merozoites

by ***Michelle Boyle[1], Danny Wilson[2] and James Beeson[1]***
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This protocol describes a method to use isolated viable merozoites for studies of erythrocyte invasion, testing invasion-inhibitory antibodies and compounds, and for imaging studies of merozoite invasion. The protocol is developed from Boyle, Wilson, et al., PNAS 2010

Materials and reagents

RPMI-HEPES standard culture media containing
- Albumax II (or heat inactivated human serum)
- glutamine
- hypoxanthine
- gentamicin

U-bottom 96 well tissue culture plate (Falcon 3077)
Human erythrocytes

Procedure

Isolate viable *P. falciparum* merozoites as described (see separate protocol; (Boyle et al., 2010))

2. Prepare 96 well plate for invasion or invasion-inhibition inhibition assays
    - Prepare RBC by washing once in culture medium and make a suspension of RBCs at 5% hematocrit
    - To each assay well add 5 μl blood (this will result in a final haematocrit of 0.5%).
    - To test compounds or antibodies for inhibition of invasion: To each assay well add 5 μl inhibitor; this will be diluted 1:10 in the final assay volume (e.g. adding 5 ul of heparin at 1 mg/ml will result in a final concentration of 100μg/ml). Include several PBS control wells spread throughout plate and a positive control (Eg. heparin and R1 peptide are effective inhibitors of invasion).
        - NOTE: if possible, include extra wells that can be smeared following invasion to check efficiency and progress of the assay. These can be included in outside wells of plates.
        - NOTE: depending on system used to agitate cultures after the addition of merozoites, invasion efficiency may not be uniform across the plate. It is recommended that merozoite invasion efficiency using different agitation methods is tested to ensure the use of wells that have similar invasion rates.
    - Plate is now ready for the addition of merozoites.
        - NOTE: concentrations and volumes can be adjusted if necessary. It has been observed that invasion is not always consistent throughout plate, perhaps due to differential agitation of wells on shaker after the addition of merozoites, therefore it is important to

spread controls across plate well and to ensure that the position of samples is modified in repeat experiments.
- NOTE: For assays that require an extended period of incubation with the inhibitor the RBCs should be added after incubation of the inhibitor with the merozoites and concentrations of drug modified accordingly.

3. Isolation of merozoites and addition to invasion assay plate
   - Merozoites are isolated from schizonts by membrane filtration as described elsewhere
   - For isolation of merozoites, prepare filter by removing the plunger from a 10 ml syringe and attach to 1.2 μm filter unit.
     - NOTE: Manufacturer's instructions for 1.2 μm filter indicate that the use of syringe units smaller that 10 ml can result in pressures that may damage the filter unit.
   - Prepare 96 well plate by tapping to resuspend RBCs and any inhibitors present.
   - Add schizonts to syringe, add plunger and filter merozoites at an even speed into a pipetting tray.
   - Add 40 μl of merozoite suspension to each well of the 96-well plate, mix well either by pipetting or tapping.
   - Place plate in 37 °C incubator on shaker (500 rpm) for 10 minutes.
   - Transfer plate to gassed culture box for 20 minutes to allow all invasion events to occur.
   - Wash cells in all wells of the plate twice with 200 μl of culture media by pelleting cultures at 1200 rpm, for 2 mins, removing supernatant, and adding wash medium.
   - Resuspend cultures in 50 μl of fresh culture media and return to incubator until analysis.
   - If extra wells have been included to monitor assay progress, smear parasites and check for newly invaded rings to assess invasion efficiency.

4. Analysis of parasitemia and determining invasion rates

   Preferred analysis of parasitemia is by flow cytometry
   - Culture parasites in 96-well plates for 36-40 hours.
     - NOTE: It is possible to count parasitemia by flow cytometry at earlier time points, however it becomes more difficult to distinguish parasites from RBC with bound merozoite and merozoite debris.
   - Stain culture by adding 100μl of EtBr 10 μg/ml to each well
     - NOTE: other staining methods are possible, for example SYBRGreen (1:5000 dilution)
   - Incubate in dark for 30-45 mins.
   - Pellet culture in centrifuge 1200 rpm, 2 mins and remove supernatants.
   - Resuspend cells in 200 μl of PBS, analyse by flow cytometry (EtBr is fluorescent in FL2).
   - Express invasion data relative to controls.

5. Preparing invading merozoites for microscopy
   Merozoites can be fixed during the process of erythrocyte invasion to enable imaging of invasion events and interactions by immunofluorescence microscopy, or electron microscopy
   - Prepare invasion assays using a mixture of RBCs and merozoites as described above
   - After mixing freshly isolated merozoites and RBCs together, incubate cells on a shaker for 2 mins. This timing is optimal for maximizing the capture of merozoites in the process of erythrocyte invasion. However, longer or shorter incubation times can be used if other events are being studied
   - Pellet cells by centrifugation and remove supernatant
   - Fix cells:
     - For immunofluorescence microscopy: resuspend cells in 4% formaldehyde with 0.0075% glutaraldehyde, then mounted on glass slides for staining
     - For EM, cells were fixed with 1% glutaraldehyde, then embedded and processed using established methods. Refer to (Riglar et al., 2011) for comprehensive methods on imaging of invading merozoites.

Further Notes

1. Parasitemias of 5-10% routinely result from described assay set up, however with highly synchronised parasites and optimized assays up to 60% parasitemia has been achieved
2. Survival of merozoites over time is dependent on a number of variables however it is longest when merozoite kept at room temperature at high concentrations
3. When gating parasites for flow cytometry, care must be taken to ensure debri from non-invaded merozoites and RBC with merozoite attached, but not invaded are excluded.

References

Boyle, M.J., D.W. Wilson, J.S. Richards, D.T. Riglar, K.K.A. Tetteh, D.J. Conway, S.A. Ralph, J. Baum, and J.G. Beeson. 2010. Isolation of viable *Plasmodium falciparum* merozoites to define erythrocyte invasion events and advance vaccine and drug development. *Proceedings of the National Academy of Sciences*. 107:14378–14383.

Riglar, D.T., D. Richard, D.W. Wilson, M.J. Boyle, C. Dekiwadia, L. Turnbull, F. Angrisano, D.S. Marapana, K.L. Rogers, C.B. Whitchurch, J.G. Beeson, A.F. Cowman, S.A. Ralph, and J. Baum. 2011. Super-resolution dissection of coordinated events during malaria parasite invasion of the human erythrocyte. *Cell Host Microbe*. 9:9–20.

XI:H. Phenotyping erythrocyte invasion using two-colour flow cytometry

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Background

The following protocol uses two-color flow cytometry to distinguish parasite invasion from parasite growth. This approach has the advantage of increasing the potential for scale-up by eliminating parasite purification or pre-treatment steps, and minimises handling of parasite cultures, which is important for less robust samples such as field isolates. Target cells (such as those from different human genetic backgrounds, or those that have had one or more receptors removed using enzymatic treatment, as detailed below) are pre-labeled with intracellular dyes CFDA-SE or DDAO-SE, then incubated with *P. falciparum* parasites. After invasion has occurred, parasites that have invaded either labeled or unlabeled cells are detected with fluorescent DNA-intercalating dyes Hoechst 33342 or SYBR Green I. The assay is sensitive, operates in a 96-well format, and can be used to quantitate the impact of either natural or experimental genetic variation on erythrocyte invasion efficiency.

The procedure detailed below includes only the DDAO-SE/SYBR Green I dye combination, which can be used with the laser combinations available on most cytometers. A more detailed standard operating procedure for the assay detailing both this and other dye combinations can be found in the resources section at the Sanger Institute Malaria Programme website:
http://www.sanger.ac.uk/research/projects/malariaprogramme-rayner

Equipment

P. falciparum culture facilities
Pipette controller (for serological pipettes)
Single-channel (1-10µL, 20-200µL and 100-1000µL) and multichannel (20-200µL) pipettes
Microcentrifuge (for microfuge tubes) and centrifuge (for tubes and plates)
Rotator
37°C incubator
Incubator culture chamber
Flow cytometer with 2 laser lines (488nm and ~635nm) and at least 2 fluorescence detectors (~520nm for SYBR Green I and ~660nm for DDAO-SE)

Reagents

CellTrace Far Red DDAO-SE (Life Technologies)
RPMI 1640 (Life Technologies)
Plasmodium falciparum culture media with (complete) or without (incomplete) 10% human sera
Neuraminidase from *Vibrio cholera* (Sigma-Aldrich)
Trypsin from bovine pancreas (Sigma-Aldrich)
Chymotrypsin from bovine pancreas (Sigma-Aldrich)
PBS (Sigma-Aldrich)
Formaldehyde (Thermo Scientific)
Glutaraldehyde (Sigma-Aldrich)

Triton X-100 (Sigma-Aldrich)
Ribonuclease A (Sigma-Aldrich)
10,000X SYBR Green I (Life Technologies)

Procedure

DAY 1

Part A – Labeling target RBC with DDAO-SE

- Prepare 3mL of a 2% hematocrit RBC suspension in RPMI in a 15mL tube
- Centrifuge the tube at 450g for 3min and discard the supernatant
- Resuspended the cell pellet with 3mL of 10μM DDAO-SE and mix the suspension thoroughly
- Incubate the cells at +37°C for 2h on a rotator
- Centrifuge the tube at 450g for 3min and discard the supernatant
- Wash the cell pellet once with complete media
- Resuspend the cell pellet with 3mL of complete media and incubate the suspension at +37°C for 30min on a rotator
- Centrifuge the tube at 450g for 3min and discard the supernatant
- Wash the cell pellet twice with incomplete media
- Resuspend the cell pellet with 3mL of incomplete media

Part B – Enzymatic treatments

- Aliquot 400μL of stained RBC into 6 microfuge tubes labeled “A” to “F”. A = Positive control (no treatment); B = Neuraminidase; C = Low trypsin; D = High trypsin; E = Neuraminidase + High Trypsin (negative control); F = Chymotrypsin.
- Add 8μL of 1U/mL neuraminidase in RPMI to tube B and tube E
- Incubate the tubes at +37°C for 1h on a rotator
- Centrifuge the tubes on a benchtop microcentrifuge for 30s and discard the supernatants
- Wash the cell pellets once with incomplete media
- Resuspend the cell pellets with 400μL of incomplete media
- Add 2μL of 10mg/mL trypsin in RPMI to tube C, 40μL of 10mg/mL trypsin in RPMI to tube D and tube E, and 40μL of 10mg/mL chymotrypsin in RPMI to tube F
- Incubate the tubes at +37°C for 1h on a rotator
- Centrifuge the tubes on a benchtop microcentrifuge for 30s and discard the supernatants
- Wash the cell pellets twice with incomplete media
- Resuspend the cell pellets with 400μL of complete media

Part C – Plate setup and incubation

- Prepare a 2% hematocrit suspension of synchronized ring-staged parasitized RBC
- Add 50μL of parasitized RBC to 18 wells of a 96-well round-bottom plate
- Add 50μL of DDAO-labeled RBC from each of the 6 tubes to 3 wells containing parasitized RBC, so that there are triplicate wells of parasites with each enzymatic treatment condition

- Mix the content of the wells and add 100µL of PBS to each of the remaining empty wells
- Place the plate inside an incubator culture chamber and gas the chamber for 3min with a mixture of 1% O2, 3% CO2 and a balance of N2
- Place the chamber inside an incubator at +37°C and incubate for 48h

DAY 3

Part D – Parasite staining with SYBR Green I

- Centrifuge the assay plate at 450g for 3min and discard 50µL of supernatant from each well
- Wash the wells with 200µL of PBS
- Add 200µL of 2% formaldehyde/0.2% glutaraldehyde in PBS to each well, mix and incubate at +4°C for 1h
- Centrifuge the plate at 450g for 3min and discard 200µL of supernatant from each well
- Wash the wells with 200µL of PBS
- Add 200µL of 0.3% Triton X-100 in PBS to each well, mix and incubate at room temperature for 10min
- Centrifuge the plate at 450g for 3min and discard 200µL of supernatant from each well
- Wash the wells with 200µL of PBS
- Add 200µL of 0.5mg/mL ribonuclease A in PBS to each well, mix and incubate at +37°C for 1h. Ribonuclease treatment is necessary to reduce background caused by low-level binding of SYBR Green I to RNA.
- Centrifuge the plate at 450g for 3min and discard 200µL of supernatant from each well
- Wash the wells with 200µL of PBS
- Add 200µL of 1:5,000 SYBR Green I in PBS to each well, mix and incubate at +37°C for 1h
- Centrifuge the plate at 450g for 3min and discard 200µL of supernatant from each well
- Wash the wells 3 times with 200µL of PBS
- Add 200µL of PBS to each well and mix to resuspend the cell pellets

The samples can now be acquired with an appropriate flow cytometer. An additional 1:100 dilution in PBS is recommended to prevent clogging the flow line. A minimum of 100,000 events should be acquired to ensure that at least 50,000 target cells can be analyzed.

Reference
Theron M, Hesketh RL, Subramanian S, Rayner JC. 2010. An adaptable two-color flow cytometric assay to quantitate the invasion of erythrocytes by *Plasmodium falciparum* parasites. Cytometry A 77(11):1067-74.

XI: I. SYBR Green I®-based parasite growth inhibition assay for measurement of antimalarial drug susceptibility in *Plasmodium falciparum*.

by ***Carmony L. Hartwig[1], Amel O. A. Ahmed[2], Roland A. Cooper[3] and Timothy T. Stedman[2]***

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Background: The purpose of this protocol is to determine the half-maximal inhibitory concentration (IC_{50}) of selected antimalarials against *Plasmodium falciparum* strains using a standardized fluorescent SYBR Green I®-based 96 microplate assay. *Adapted and standardized based on methods outlined in Bennett et al. (2004) and Smilkstein et al. (2004).*

Apparatus

Class II A/B3 biosafety cabinet (BSC)
Denver APX-60 analytic balance (or equivalent, d=0.1mg)
Modular incubator chamber (Billups Rothenberg)
37°C (5% CO_2) incubator
Clinical centrifuge and microcentrifuge
Microplate reader (Spectramax M2, Molecular Devices, or equivalent set with an excitation of 490 nm and emission detection at 540 nm)
Water bath (37°C)

Equipment

25 cm^2 cell culture flasks
25 mm syringe filter (0.22 micron)
20 ml syringe
Conical Falcon centrifuge tubes, 15 ml and 50 ml
96 micro-well flat bottom plates
96 deep-well flat bottom plates
Mat lids for 1.0 ml 96 deep-well plates
1.5 ml black microcentifuge tubes
Pipets, 1000 µl, 100 µl and 10 µl
Pipet tips, 1000 µl, 100 µl and 10 µl
Multichannel pipet, 300 µl
Sterile, barrier pipet tips, 300 µl, 100 µl and 10 µl
Sterile reservoir basins
Parafilm
Aluminum foil

Materials and reagents

Parasite culture (preferred 5% parasitemia, and at least 70% ring stage)
Complete culture media (CM)
- 10% human serum type A*
- RPMI 1640(with folate, or folate-free depending on antimalarial test)
- 0.2% D-glucose

0.2 mM hypoxanthine
2 mM L-glutamine
0.25% sodium bicarbonate
25 mM HEPESbuffer
50mg/ml gentamycin reagent solution

**Albumax I or II (Invitrogen) may be substituted for human serum; however, assays must be consistently performed using either serum- or Albumax-supplemented media as increased IC_{50} values result when human serum is substituted with Albumax II, particularly for artemisinin.*

Human blood type O^+
RPMI 1640 media, folate-free
RPMI 1640 media, with folate
Double-distilled water (ddH_2O)
DMSO
70% ethanol
Blood gas mixture for purging modular incubators and parasites cultures (90% N_2, 5% CO_2, 5% O_2)
SYBR Green I® (SGI; 10,000X in DMSO, Invitrogen)
2X Lysis buffer

20 mM Tris
5 mM EDTA
0.008% wt/vol saponin
0.08% vol/vol Triton X-100
q.s. to 1 L with ddH_2O (~977 ml)
pH to 7.4-8.0
Store at RT and dilute to 1X working stock with ddH_2O prior to plating.

Artemisinin (ART; 10 mM) drug stock

Dissolve 0.0282 g of ART (MW 282.34 g/mol) in 10 ml of 70% ethanol.

Chloroquine diphosphate (CQ; 10 mM) drug stock

Dissolve 0.0516 g of CQ (MW 515.90 g/mol) in 10 ml of sterile ddH_2O (or70% ethanol).

Quinine (QN; 10 mM) drug stock

Dissolve 0.0324 g of QN (MW 324.42 g/mol) in 10 ml of 70% ethanol.

Cycloquanil (CYC; 10 mM) drug stock

Dissolve 0.0288 g of CYC (MW 288.18 g/mol) in 10 ml of DMSO.

Sulfadoxine (SDX; 100 mM)drug stock

Dissolve 0.310 g of SDX (MW 310.33 g/mol) in 10 ml of DMSO.

Pyrimethamine (PYR; 10 mM) drug stock

Dissolve 0.0249 g of PYR (MW 248.71 g/mol) in 10 ml of 70% ethanol.

For all drug stocks, syringe filter, aliquot and store at -20°C.

Drug compounds may be substituted or added to the assay as desired. Dilutions for dose – response curves should aim to center the IC_{50} response in sensitive strains, and capture the entire effective concentration range of the drug tested to include the lower and upper limitend points on the curve.

Preparation

Day 1

For assay set-up and parasite plating, work in a BSC and use sterile equipment. SGI incubation steps and spectrophotometric quantification may be performed on a bench-top surface using non-sterile materials.

Preparation of Drug Masterplate (DMP)

1. Based on estimates of IC_{50} values, starting concentrations for each drug should center the dose-response curves within each row. From the DMP to the assay plate (AP) a 1:4 dilution of drug is made. However, all drugs concentrations are made up as 8X working stocks to account for a 2X dilution to double the final DMP volume.

    A. *NOTE:* For CQ, ART, QN and CYC, RPMI and CM containing folate is used. For SDX and PYR screening, folate-free media (RPMI-*FF* and CM-*FF*) is used. Make up a separate DMP for each drug set using the appropriate media supplement (See diagrams below, **DMP-1** and **DMP-2**).

2. In a BSC, make 8X working drug stocks by diluting freezer stocks with appropriate volume of RPMI in 15 ml conical tubes as follows:

    A. CQ, QN and CYC (10 mM stock) – Add 20 µl in 10 ml RPMI.

    B. ART (10 mM stock) – Add 2 µl in 10 ml RPMI.

    C. SDX-H (100 mM stock) and PYR-H (10 mM stock) – Add 400 µl in 4.6 ml RPMI-*FF*.

    D. SDX-L(100 mM stock) – Add 5 µl in 5 ml RPMI-*FF*.

    E. PYR-L (10 mM stock) – Add 40 µl in 5 ml RPMI-*FF*.

    F. NOTE: The “H” in SDX-H and PYR-H refers to the drug prepared at a higher concentration; “L” in SDX-L and PYR-L to drug prepared at a lower concentration.

DMP-1 : Concentrations for CQ, ART, QN and CYC in RPMI

4X initial concentration from starting well of AP

Antimalarial		1	2	3	4	5	6	7	8	9	10	11	12	
CQ	A	**20 µM**	1:2 serially dilution in RPMI →										**RPMI ONLY** (No Drug Control)	A
	B	(500 µl)	(250 µl:250 µl CM)											B
ART	C	**2 µM**	1:2 serially dilution in RPMI →											C
	D													D
QN	E	**20 µM**	1:2 serially dilution in RPMI →											E
	F													F
CYC	G	**20 µM**	1:2 serially dilution in RPMI →											G
	H													H

DMP-2 : Concentrations for SDX – H, SDX – L, PYR – H and PYR – L in RPMI-*FF*

4X initial concentration from starting well of AP

Antimalarial		1	2	3	4	5	6	7	8	9	10	11	12	
SDX - H	A	**8000 µM**	1:2 serially dilution in RPMI →										**RPMI-*FF* ONLY** (No Drug Control)	A
	B	(500 µl)	(250 µl:250 µl CM-*FF*)											B
SDX - L	C	**500 µM**	1:2 serially dilution in RPMI →											C
	D													D
PYR - H	E	**800 µM**	1:2 serially dilution in RPMI →											E
	F													F
PYR - L	G	**80 µM**	1:2 serially dilution in RPMI →											G
	H													H

3. Place 500 µl of each prepared drug working stock in RPMI (or RPMI-*FF)*into starting wells of column 1, in duplicate rows for each drug or drug concentration, in labeled 96 deep-well plates.

4. Pipette CM (or CM-*FF*) into a sterile reservoir trough and transfer 250 µl of CM into all wells of plate, columns 2-12, using the multichannel pipette equipped with 300 µl pipet tips.

5. Serially dilute drugs in plate columns 2-11 by removing 250 µl of drug from column 1 and pipetting into column 2. Mix and remove 250 µl from column 2 and add to column 3. Repeat through column 11, and discard remaining 250 µl from column 11. **Do not add drug to column 12.**

A. *NOTE*: Column 12 is designated for RBC and culture growth controls (CGC).

6. Following serial dilution, add 250 µl of CM (of CM-*FF*) to the plate in columns 1-12, so final volume is uniformly 500 µl in all wells. Your DMP is now 4X the concentration of your drug AP.

A. Use immediately or cover DMPs with mat lid and seal with aluminum foil to keep sterile. Label, initial and date. Place in 4°C until ready to use. Discard after 8 days, or 9 assays.

Day 2

Preparation of Drug Assay Plate (AP)

7. If using a cryopreserved culture, thaw the *Plasmodium falciparum* isolate and cultureusing standard techniques (see “Thawing of cryopreserved *Plasmodium falciparum*” methods within this volume).

8. Maintain the parasite culture at 5.0% hematocrit. Change growth medium as necessary and grow the parasite culture for one to two full growth cycles until the culture (preferably) attains 5-10% ring stage parasitemia (must be greater than 1.0% parasitemia to run in assay).

9. To put the culture into a drug assay, make a blood smear to calculate parasitemia. If culture is less than 70% ring stage, synchronize the parasite culture to ring stage using a standard sorbitol synchronization procedure (Lambros and Vanderberg, 1979).

A. NOTE*:* If sorbitol treatment is necessary, synchronize and adjust to 5% hematocrit then return culture to incubator for ~2 h prior to plating with drug. Make another blood smear to calculate parasitemia and make sure that the rings look healthy.

10. Prior to putting culture into the assay plate, bring culture volume back up to 5 ml in CM.

A. NOTE*:* If running assays for SDX or PYR, make sure to resuspend cultures in the CM-*FF* at this point.

B. Calculate and dilute the culture to 0.5% parasitemia at 2% hematocrit in a 50 ml conical falcon tube or 100 ml sterile basin using the following equations:

1. Culture: 1600 µl/ (%P/2) = X µl parasite culture needed per plate (CPP).
2. Blood: 320 (%H) – (CPP/10) = X µl blood needed per plate (BPP).
3. CM: (16000 µl – CPP– BPP)/1000= X ml CM needed per plate.
   a. NOTE: %H refers to the percent hematocrit of blood in the AP and %P refers percent parasitemia of the culture used in the assays. 320 is a factor for calculating the BPP.

11. Preparation of **AP**.

A. Remove DMP from 4°C if stored. Allow ~ 30 min for plates to come up to RT.

B. Linearly transfer 50 µl of 'no drug' control from DMP column 12 to column 12 of flat-bottom, sterile, 96-well AP. Continue the linear transfer of columns 11 to 1 from the DMP to the AP, starting from the lowest concentration (column 11) to highest concentration (column 1) of drug, making sure to change tips between each AP.

C. Transfer culture prepared from step 10.B.1 to a sterile reservoir trough.

D. Add 150 µl of mixed parasite culture to control wells column 12 rows C-E first (see schematic below). Continue adding parasite culture to each column of the assay plate starting from lowest drug concentration to highest drug concentration (columns 11 through 1).

1. NOTE: Do not add culture to control RBC wells in column 12, rows F-H.

AP Layout for Parasite Culture Dilutions with DMP

	1	2	3	4	5	6	7	8	9	10	11	12	
A	**50 µl DMP : 150 µl culture**											**Blank (RPMI only)**	A
B													B
C													C
D												**iRBC (RPMI)**	D
E													E
F												**RBC 2% H (RPMI)**	F
G													G
H													H

E. Make a 2% hematocrit solution for negative controls (column 12, rows F-H). Add 150 µl of a 2% hematocrit solution to control wells. Place top on the AP and label with the strain ID, date, time and initials.

F. Place plate(s) in modular incubation chamber containing 2 ports and place a sterile microassay plate with ddH_20 at the bottom of modular incubation chamber to maintain humidity levels.

1. Open both ports to the modular chamber; connect high nitrogen mixed gas (5% CO_2, 5% O_2, and 90% N_2) to one port and gas for at least 2 minutes. Close the opposing gas port then seal the source gas port no more than 5 seconds later (pressure may build otherwise). Do not overpressurize incubators.

2. Place incubation chamber in 37°C CO_2 incubator for 72 h. Incubators can be provided with fresh blood gas every 24 h for consistency.

12. Remove plates from the incubator after 72 h (± 1 h). Seal drug plate(s) with aluminum foil (or parafilm) and freeze the drug plate(s) at -20°C until frozen solid.

NOTE:Plates may be stored in -20°C long term until fluorescent detection can be performed, tested up to two weeks. Plates should be tightly sealed to avoid sublimation.

Day 3

SGI Detection of AP

13. To process for SGI detection of parasite growth, thaw the frozen plates in the 37°C incubator for 1 h. While plates are thawing, prepare the 1X SGI Lysis buffer (LB).

14. Measurement of SGI-intercalated parasite DNA.

A. Calculate the total volume of 1X LB needed for the number of plates to be analyzed.

1. You will need ~10 ml of 1X LB for each plate run. To ensure that you make enough 1X LB for transfer of all plates, make up at least 2 ml more than the exact calculation (*ex.* 2 plates: (2 x 10 ml) + 2 ml = 22 ml 1X LB).

2. Dilute 2X LB (see Reagents above) to 1X LB with ddH_2O prior to adding SGI (10,000X stock). Apply 4X (0.4 µl/ml final volume) of the SGI solution to the 1X LB.

a. NOTE: SGI is light sensitive. All steps for SGI detection should be performed under reduced lighting.

3. Add 100 µl of SGI in 1XLB to each well of a fresh (non-sterile) 96-well plate. You may wish to use non-transparent white or black 96-well microplate specifically designed to read fluorescence.

4. Add culture test samples from thawed AP with proper controls to your prepared SGI 1X LB plate.

a. Transfer cultures starting with columns 1 (least parasite growth) and continue down columns 2-11 by mixing gently and removing 100 µl

of culture from the AP and layering in top of the SGI 1X LB plate. 300 µl pipet tips are preferred to give additional room to accommodate any bubbles within the tips. Try to avoid foaming.

b. Change tips and transfer control column 12, designated for RBC and culture growth controls.

5. Seal SGI plate with aluminum foil, or place in a dark drawer, and incubate for 1 h at room temperature in the dark.

B. Measure fluorescence associated with SGI-intercalated parasitic DNA at 490 nm excitation and 540 nm emission in the Spectramax M2 Microplate Reader or an appropriate spectrophotometer capable of reading 96-well formats.

15. Analyze the raw data output using Prism v5.0 (GraphPad Prism, San Diego CA) or equivalent type of analysis program to construct growth inhibition curves from normalized data to determine IC_{50} values for all compounds tested.

A. Raw data acquired from the spectrophotometer must be adjusted by subtracting the averaged value of the RBC controls from the iRBC wells treated with drug for each plate analyzed. The iRBC wells with no drug serve as a positive control for parasite growth.

B. Once the adjusted data values are obtained, they should be pasted into a Prism v5.0 file (or equivalent) and normalized in a separate analysis sheet. Normalized values should be plotted by antimalarial treatment and used to generate the IC_{50} values for parasite growth inhibition in the presence of each compound tested.

C. All analyses are run in triplicate; therefore, each antimalarial must be tested in three individual plate runs, in duplicate rows of antimalarial exposure, for each AP. This ensures optimal accuracy on the AP and accounts for variability between runs for a given *P. falciparum* strain.

16. Once growth curves have been generated, ensure the adjusted R^2 is at least 0.90, and the 95% confidence interval scorresponding to the top and bottom of curves contain 100% and 0%, respectively. Obtain the IC_{50} from the Prism v5.0 (or equivalent) analysis output, and calculate the SEM value from the IC_{50} and Log SE.

References

Bennett, T. N., M. Paguio, B. Gligorijevic, C. Seudieu, A. D. Kosar, E. Davidson, and P. D. Roepe. 2004. Novel, rapid, and inexpensive cell-based quantification of antimalarial drug efficacy. Antimicrob. Agents Chemother. 48:1807–1810.

Lambros, C. and J. P. Vanderberg. 1979. Synchronization of *Plasmodium falciparum* erythrocytic stages in culture. J. Parasitol 65:418–420.

Smilkstein, M., N. Sriwilaijaroen, J. X. Kelly, P. Wilairat, and M. Riscoe. 2004. Simple and inexpensive fluorescence-based technique for high throughput antimalarial drug screening. Antimicrob. Agents Chemother. 48:1803–1806.

XII. Quantification of hemozoin or malaria pigment in tissues

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A) Hemozoin quantification in organ cryosections by densitometric analysis

Equipment

Light microscope equipped with a 20x objective and a camera
Software for densitometric analysis (e.g. Axiovision 4.6, Zeiss)

Materials and reagents

Unstained organ cryosections with a thickness of e.g. 7 µm. The organs should be preferably perfused to remove circulating parasites, as these may also contain hemozoin.

Procedure

- Take multiple transmitted light images of the unstained organ cryosections through a 20x objective lens (e.g. two rows of three consecutive fields)
- Perform densitometric analysis on the images and subtract background
- Calculate the relative quantity of hemozoin/µm^2 tissue

B) Hemozoin quantification by heme-enhanced luminescence

The advantages of this method (compared to method A) are: higher sensitivity and higher throughput.

Equipment

tissue homogenizer (e.g. Precellys Lysing kit)
1.5 mL Eppendorf tubes (compatible with sonicator)
benchtop centrifuge
incubator
vial sonicator (e.g. VialTweeter, Hielscher)
96-well plates suited for luminescence (e.g. Perkin Elmer)
multichannel pipette
luminometer (plate reader, e.g. Thermo Luminoskan Ascent)

Materials and reagents

Perfused organs
Homogenization solution (Solution A)
50 mMTris/HCl (pH 8.0), 5 mM $CaCl_2$, 50 mMNaCl, 1% Triton X-100
Proteinase K from *Tritirachium album*
(buffered aqueous glycerol solution, >800 units/mL, CAS number 39450-01-6, e.g. from Sigma)
Washing solution (Solution B)
100 mM $NaHCO_3$ (pH 9.0), 2% SDS
Heme/hemozoin dissolving solution (Solution C)
100 mM NaOH, 2% SDS, 3 mM EDTA
Buffering solution (Solution D)
1 M Na_2CO_3 (pH 10.4)
Working solution
4 volumes solution C + 1 volume solution D

Hematin stock solution
2 mM hemin (CAS number 16009-13-5,e.g. from Sigma) in solution C (Store at -20°C)
Luminol (3-aminophthalhydrazide 97%, CAS number 521-31-3, e.g. from Aldrich) stock solution
20 mM luminolin dimethyl sulfoxide (Store at -20°C)
tert-butylhydroperoxide solution (70 wt % in H_2O, CAS number 75-91-2, e.g. from Aldrich) (Store at 4°C)

Procedure

A) Isolation of hemozoin from organs

- Homogenize the tissue sample in five volumes (e.g. 500 µL/50 mg tissue) of solution A (e.g. with a Precellys homogenizer). Weight of the tissue sample should be 30-60 mg (e.g. for mouse liver, spleen, kidney or lung), for mouse brain and heart tissues ~ 200 mg is required.
- Transfer the homogenate into a 1.5 mL Eppendorf tube
- Supplement the homogenate with 1% v/v proteinase K (e.g. add 1 µL proteinase K solution per 100 µL homogenate) and incubate overnight at 37°C
- Sonicate for 1 min (10 W, pulse 0.5 sec)
- Centrifuge at 11,000 x g for 45 min
- Discard the supernatant and wash the pellet in 700 µL of solution B with subsequent sonication (1 min, 10 W, pulse 0.5 sec) and centrifugation (11,000 x g for 30 min)
- Repeat this washing step 3 times
- Dissolve pellet in 100 µL of solution C and sonicate(1 min, 10 W, pulse 0.5 sec)
- Centrifuge 30 min at 11,000 x g
- Store the extracted hemozoin sample at room temperature until luminescence measurement

B) Hematin quantification

- Dilute hematin stock solution (2 mM) 100x in solution C (final concentration 20 µM). From here keep reagents at room temperature to prevent SDS precipitation
- Add 50 µL working solution to each well of the 96-well plate
- Make two series of serial dilutions of hematin (10 µM-1.2nM) by adding 50 µL of 20 µM hematin solution in well A1 and A2
- Make eight serial dilutions (e.g. 2/3, 1/2 or 1/3) of the extracted hemozoin samples (use 1 microtiter plate column/sample). Testing the appropriate dilution for each sample type is crucial since too concentrated samples may result in false negative results by luminol conversion before the measurement.
- Reserve at least two wells as a negative control (blank)
- Dilute luminol stock solution (20 mM) 35x in working solution
- Dilute peroxide stock solution (70%) 10x in working solution
- Go to the luminometer and adjust the settings (shake plate during 5 sec, read 1 sec/well, start the readings always from the top of the column: A1→B1→…→H1→A2→B2→…→H2→A3→…)
- Add 100 µL of the diluted luminol solution with a multichannel pipette (column-wise)
- Add 100 µL of the diluted peroxide solution with a multichannel pipette (column-wise) with an interval of 9 sec between the columns (stick to the time schedule to keep the time deviation between the luminescence measurements of the individual wells as small as possible)

- Bring the plate in the luminometer and start the reading as fast as possible
- Subtract the background luminescence of the blank sample from the measurements
- Calculate the unknown hemozoin concentration in the sample from the calibration curve of the hematin concentration (nM, logarithmic scale) on the X-axis *versus* the blank-subtracted luminescence signal (events/sec) on the Y-axis. Use only the measurements within the linear part of this curve.
- Calculate the amount of Hz/mg tissue or organ

Reference

Deroost K, Lays N, Noppen S, Martens E, Opdenakker G, Van den Steen PE. 2012. Improved methods for haemozoin quantification in tissues yield organ- and parasite-specific information in malaria-infected mice. Malar J 14;11:166.

MOSQUITOES AND PARASITES

I. Rearing of *Anopheles stepheni* mosquitoes

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See MOSQUITOES AND PARASITES, section IV:D, page 147

II. Sugar-feeding preference method

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Equipment

assay cage
dissection microscope

Materials and reagents

food dyes
test solutions
commercially available food dyes
Kleenex paper

Procedure

- Pick pupae for two (or 4 or 6 or …) parallel experiments at a time and place them in assay cages (or divide the adults into groups and place in assay cages).
- On the day of the experiments, starve the mosquitoes in an environment of lower humidity and higher temperature than normal rearing conditions for seven hours. This is done to increase the number of mosquitoes that feed
- Prepare the two test solutions and add the food dyes. One colour to each solution and change the colour in the two cages: i.e. solution A is green and solution B is red in the first cage and in the second cage solution A is red and solution B green. We used 10 drops of commercially available food dyes (Hushållsfärg, Ekströms, Sweden) to 5-10 mLof test solution. These dyes are bought in solutions, which contain water, glycerol, ethanol and red (E120) or green (E104, E131) dyes. It is probably ok to use other food colourings but make sure to test the colour preference first by having solution A = solution B apart from the colour.
- Place a Kleenex paper in each test solution and place the test solutions in the cage at a set distance for a period of 2-3 hours. In our experiments starvation and feeding were performed during the 12h photoperiod, however, the lights were turned off during the feeding period.

- Kill the mosquitoes and count the number of mosquitoes that has fed on each solution using a dissection microscope.

Preference method separating olfactory and tactile/taste responses
Performed as above but the Kleenex paper that the mosquitoes fed from is placed in an outer cup containing sugar solution and the test solution is placed in an inner cup inaccessible for the mosquitoes.

III. Procedures required to generate ***Anopheles stephensi*** mosquitoes infected with the human malaria parasite, ***Plasmodium falciparum***

by ***Martin Looker***[1] *and* ***Andrew W. Taylor-Robinson***[2]
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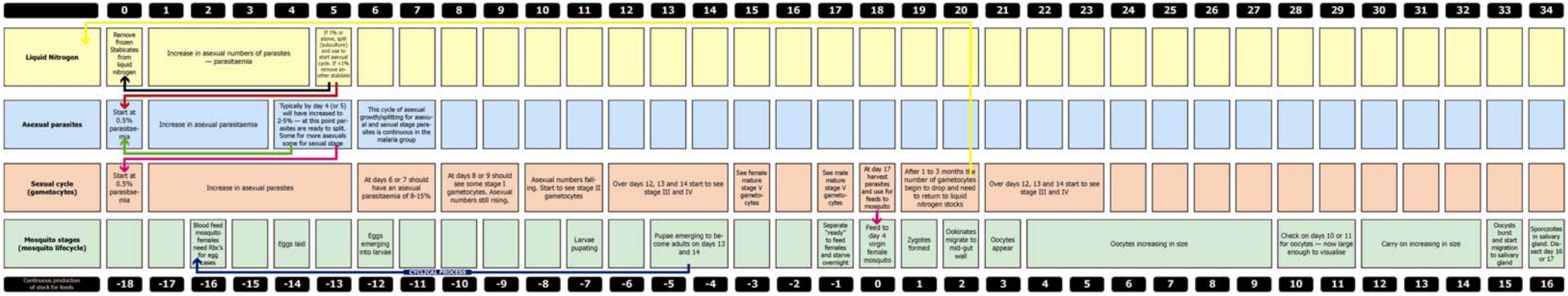
The production of *Anopheles stephensi* mosquitoes infected with the human malaria parasite, *Plasmodium falciparum,* involves the propagation of parasite and vector life cycles in parallel. It is a technically difficult procedure which requires considerable pre-planning and attention to detail in order to be successful. In particular, the precise timing of each stage of the process is of paramount importance to ensure a consistant supply of infected mosquitoes for the purposes of research. This 'timeline' is a valuble organisational tool in that it identifies, in short-hand form, precisely what tasks need to be performed on a day-to-day basis. In our laboratory, we have found this schematic representation to be useful not only as an aide memoire for the experienced parasitologist but also as a teaching aid to researchers new to malaria.

Schematic representation of the practical procedures and chronological order required to generate *Anopheles stephensi* mosquitoes infected with the human malaria parasite, *Plasmodium falciparum*.

For ease of examination of the detail in this timeline, the image above may be magnified within the Microsoft Word document. Alternatively, click on the link below to see a printable pdf version (Adobe Acrobat Reader required) or e-mail the authors to receive a pdf or jpg file.



IV. Culturing of sexual, oocyst, and sporozoite stages

IV:A. *Plasmodium falciparum* gametocyte culture, purification, and gametogenesis

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Equipment

incubator (37 °C)
centrifuge
culture flasks (25-cm^2, 75-cm^2, and 150-cm^2, Corning, Canted neck flasks)

Materials and reagents

human RBC washed in incomplete medium
incomplete medium
RPMI 1640
25 mM HEPES
0.37 mM hypoxanthine (pH 7.4)
complete medium
incomplete medium (above) containing 10% heat-inactivated pooled normal human serum
29 mM $NaHCO_3$
donor culture (intra-erythrocytic parasite culture) maintained below 5% parasitemia by routine subculturing
60% Percoll:
9 volumes of Percoll stock
1 volume of 10× RPMI
5 volumes of 1× RPMI
exflagellation-inducing medium:
10 mM Tris (pH 7.6)
170 mM NaCl
10 mM glucose
10% heat-inactivated normal human serum
25 mM $NaHCO_3$
50 to 100 μM xanthurenic acid (optional)
Nycodenz (as step gradient of 6%, 11%, and 16%)

Procedure

- Spin down freshly drawn O^+ human blood (not more than 2 to 4 days old) in 15-mL centrifuge tubes at 1,000 rpm for 10 min. Discard the supernatant including the WBC containing buffy coat. Wash the RBC pellet 3 to 4 times using incomplete medium and resuspend it with an equal volume of incomplete medium to give rise to a final suspension of 50% RBC.
- Start cultures for gametocytes at 6% hematocrit and 0.3% parasitemia. Example: In a 75-cm^2 flask, add 1.8 mL of 50% washed RBC, estimated volume of parasites from a donor culture, and complete medium to make up the volume to 15 mL.
- Change medium (12 mL) every day. Make sure that the temperature of the medium is 37 °C and culture temperature does not fall below 37 °C during the

medium change. A slide warmer set at 37 °C can be used inside the hood to maintain temperature of cultures during medium change.
- Monitor the parasite growth every other day and when parasitemia reaches 3 to 5%, start changing the medium by double the medium volume (25 mL). On the subsequent days keep changing 25 mL of medium on a daily basis.
- It takes around 14 to18 days to obtain matured gametocyte cultures.

Comments

Highly synchronous stages (I to V) of *Plasmodium falciparum* gametocytes can be obtained by N-acetyl glucosamine treatment of culture (Parasitology 93(Pt 2):263-274, 1986 and Mol Biol Pharmacol 68(1):151-154, 1994).

Enrichment using Percoll centrifugation

- Prepare the 60% Percoll.
- Spin down the parasite culture and resuspend it to ~25% hematocrit in incomplete medium.
- Carefully layer 1 volume of parasite suspension over 2 to 2.5 volumes of 60% Percoll in a 15-mL (13 × 100 mm) polypropylene or Corex glass tube.
- Centrifuge the suspension at 10,000 rpm for 20 min at room temperature.
- Collect parasites at the interphase, wash them 3 or 4 times in incomplete medium, and check them by Giemsa stain.

Gametogenesis and purification of *P. falciparum* gametes/zygotes

Mature gametocytes are stimulated to undergo gametogenesis for 30 min at room temperature (25 to 26 °C) and gametes/zygotes are purified by centrifugation.

- Resuspend mature *P. falciparum* cultured parasites at 20% hematocrit in the exflagellation-inducing medium at a pH of 8.1 to 8.3 at room temperature for 30 min. Addition of 50 to 100 μM xanthurenic acid can also enhance the parasite yield.
- Layer the parasite suspension over a discontinuous step gradient of Nycodenz (6%, 11%, 16%) and spin it at 16,000 × *g* for 15 min at room temperature.
- Gametes/zygotes sediment at the interface of 6% and 11%.

References

Lobo CA, Konings RN, Kumar N. 1994. Expression of early gametocyte-stage antigens Pfg27 and Pfs16 in synchronized gametocytes and non-gametocyte producing clones of *Plasmodium falciparum.* Mol Biochem Parasitol 68(1):151-154.

Ponnudurai T, Lensen AH, Meis JF, Meuwissen JH. 1986. Synchronization of *Plasmodium falciparum* gametocytes using an automated suspension culture system. Parasitology 93(Pt 2):263-274.

IV:B. Cultivation of *Plasmodium falciparum* gametocytes for mosquito infectivity studies

by ***Martin Looker***[1] *and* ***Andrew W. Taylor-Robinson***[2]
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Gametocytes of *P. falciparum* are established from continuously maintained cultures of asexual blood stage parasites. Gametocytes begin to form in significant numbers in blood culture only following daily dilution with fresh RBC and medium for several days. In order for gametocytes to comprise a substantial proportion of PRBC, it is necessary for asexual PBRC to reach a high density and become stressed in vitro before conversion to gametocyte production. This occurs typically 6 to 8 d after the dilution of a culture. Gametocytes then require at least a further 8 d to reach maturity. Cultures for gametocyte production are therefore maintained for a minimum of 14 d without further dilution with fresh RBC in order to obtain a large proportion of mature and infectious gametocytes. We routinely perform gametocyte feeds to mosquitoes using cultures propagated continuously for the preceding 14 to 17 d.

Materials and reagents

Refer to **Appendix** of this protocol for details of materials and generic methods.

Propagation of asexual blood cultures

- Retrieve an isolate of *P. falciparum* cloned line 3D7A from liquid nitrogen and place it in a 25-cm^2 tissue culture flask along with 0.25 mL of freshly washed RBC and 6 mL of complete medium. Gas the culture for 30 s and tighten the culture flask cap. Keep it in a dedicated 37 °C dry incubator.
- At the same time every day the culture requires a medium change. Tip the flask(s) at 30 degrees to the horizontal, which results in good separation between RBC and the medium that forms the over-layer. As flasks are settled, warm the appropriate volume of complete medium to 37 °C. Settling of flasks and warming of medium takes 40 to 60 min. Remove the spent medium from each culture, discard and replace it with an equal volume (6 mL) of fresh medium, and gas the flask for 30 s. Perform all operations on a warming plate set to 37 °C in order to minimize heat loss to cultures during the time they are out of the 37 °C incubator.

Growth of blood cultures for gametocyte production

- In order to produce gametocytes on a long term basis, it is necessary to maintain a stock of 'asexual' cultures from which the gametocyte cultures are drawn. Every 5 d, flasks which have reached or exceeded a parasitemia of 1% may be subcultured ("split") by adding fresh RBC in order to reduce the parasitemia to about 0.5%. In this way, from one flask at a high parasitemia a number of 'daughter' flasks may be started at a lower parasitemia. Some of these flasks may be used for further stock asexual cultures and some may be used for gametocyte culture. The new asexual stock cultures are grown for another 5 d before being split again when a minimum parasitemia of 1% is reached. This guarantees a continuous supply of cultures for later gametocyte preparation. Existing gametocyte cultures are not subcultured but continue to have their medium changed daily.

- After 6 to 8 d post-subculture, developmental stage I and II gametocytes will start to appear (following asexual PRBC 'stress' once a threshold parasitemia is reached) and from 14 d post-subculture flasks should contain mature, stage V, gametocytes. By 17 d,1 to 4% of PRBC should be stage V gametocytes and the gametocytemia of all stages of development (I-V) may be up to 12%. Progress can be checked by examining thin blood films taken periodically post-subculture (usually on *Days 8, 12,* and *15*).
- Although it is possible to attain much higher gametocytemias by enriching for gametocytes by a variety of treatments (Percoll or Nycodenz density gradient centrifugation or selective drug treatment are the most commonly used), this is not recommended for mosquito infectivity studies. In the experience of ourselves and others, artificially increasing the gametocytemia is often counterproductive to satisfactory transmission of the parasite to the mosquito vector.
- Development of male sexual stage parasites is more rapid than that of females, so mature male gametocytes may be seen from 14 d but mature females usually only from 16 d post-subculture. In order to ensure a good balance of male and female gametocytes for the purposes of transmission, it is common practice to mix cultures of different ages at the time of the blood feed (see MOSQUITOES AND PARASITES, section II:C).

References

Graves PM, Carter R, McNeill KM. 1984. Gametocyte production in cloned lines of *Plasmodium falciparum*. Am J Trop Med Hyg 33(6):1045-1050.

Ifediba T, Vanderberg JP. 1981. Complete in vitro maturation of *Plasmodium falciparum* gametocytes. Nature 294(5839):364-366.

Read M, Hyde JE. 1993. Simple in vitro cultivation of the malaria parasite *Plasmodium falciparum* (erythrocytic stages) suitable for large-scale preparations. Methods Mol Biol 21:43-55. [Includes useful background information on equipment, reagents, cryopreservation of parasites, preparation of thin blood films, and treatment of contamination]

Appendix

Safety precautions

- *Plasmodium falciparum* is classified as a Category 3 pathogen and all appropriate health and safety measures must be carried out when maintaining this parasite in human blood in vitro, in keeping with local regulations. Perform all work in a Class II safety cabinet. Exclude all nonessential staff from the laboratory while work is in progress. Always wear a Howie-style laboratory coat and latex gloves.
- Any isolate of *P. falciparum* that is used routinely should not be resistant to standard antimalarial drugs.
- Discard spent medium and all disposable plasticware in a hypochlorite disinfectant (e.g., Chloros or Presept) to a concentration of 10% free chlorine before disposal. Swab all working areas with 70% ethanol at the conclusion of any work. Provide a wash bottle of 70% ethanol to flood any spillage of infective material.
- Obtain blood and serum from a reliable sources only, such as your local Blood Transfusion Service, where it is prescreened for the presence of HIV and hepatitis viruses.

Complete medium

RPMI 1640 medium is purchased as sterile 1-liter bottles (Gibco, 041-91187A). This medium contains 5.96 g/L HEPES buffer, 3.60 g/L glucose, and 50.0 g/L hypoxanthine. To each 1-liter bottle, add 42 mL of freshly prepared filter-sterilized 5% sodium bicarbonate (Sigma S-5761) (this is referred to as 'incomplete' medium). Following the further addition of 100 mL of heat-inactivated pooled human serum, the liquid is now 'complete' medium. Store the medium at 4 °C and use it within 7 d, during which time the pH should be monitored (optimum pH 7.3).

Retrieval of parasites from liquid nitrogen

- Thaw cryotube(s) in a 37 °C water bath for 2 min.
- Transfer the thawed contents to a sterile microcentrifuge tube and centrifuge it at 10,000 × *g* for 1 min, then remove the supernatant.
- Resuspend the pellet in 1 mL of PBS + 10% sorbitol; add this slowly dropwise with continuous mixing.
- Repeat steps 2 and 3 twice more (i.e., total of 3 washes).
- Wash and resuspend the pellet in complete medium when the cells are ready for culture.

Washing human RBC

- Use group O, Rhesus group positive whole blood taken into adenine-CPD (citrate phosphate dextrose). Blood used should be less than 7 d old.
- Centrifuge the blood at 800 × *g* for 5 min.
- Remove the white blood cells by aspiration of the buffy coat.
- Add an equal volume of incomplete medium and centrifuge it at 800 × *g* for 5 min.
- Discard the incomplete medium.
- Repeat steps 4 and 5 twice more (i.e., total of 3 washes).
- After washing, the packed RBC may be used directly (i.e., at a 100% hematocrit) and any remainder discarded. Alternatively, the packed RBC may be resuspended in an equal volume of complete medium (i.e., at a 50% hematocrit), which should be stored at 4 °C and used within 7 d.

Gas mixture

1% oxygen, 3% carbon dioxide, and 96% nitrogen (BOC Specialty Gases, or your local specialist gas supplier). Pass the gas mixture from the cylinder via a gas-reducing valve into the safety cabinet by a silicon tube. Filter the gas through an in-line Whatman gamma 12 sterilizing unit with a 0.3-µm filter tube. Connect the filter to a sterile Millex GS 0.22-µm disc filter, the end of which is attached to a 19G × 1.4-inch blunt-ended needle fitted to a 1-mL syringe. When gassing a culture flask, the flow rate should be moderate but sufficiently strong to ruffle the surface of the liquid.

Pooled serum

Pool human group O, Rhesus group positive serum from a minimum of 10 donors to minimize variations of quality between individuals. Packs of serum are routinely heat-inactivated at the source, but if not they should be held at 56 °C for 1 h. Test the pools before full-scale use to ensure that they are sterile and support parasite growth, in particular that of gametocytes. Aliquots may be stored at −20 °C for up to 6 months.

Parasites

In order to maximize the likelihood of satisfactory gametocyte production, it is best to use a line or clone of *P. falciparum* that is known for its production of gametocytes. Good producers include NF54, 3D7A, and HB3A, obtainable from the MR4 repository. Restart cultures from frozen stocks every 2 months or less since gametocytogenesis in a continuously cultured line will start to wane after this period.

Parasite staining

Use improved R66 Giemsa (BDH 350864X) diluted 1:10 with Sorensen's buffer (HD Supplies; HDS 20, pH 7.2). Fix thin blood films in 100% methanol and stain for 20 min in the 10% Giemsa solution. Wash them with tap water for 20 s and allow to air dry before viewing by light microscopy using oil immersion at 1000× magnification.

Thin blood film

Small volume of RBC removed from culture flask and smeared across a glass slide to give a monolayer of RBC.

Sterile plasticware

25-cm^2 cell culture flasks (Iwaki 3100-025 or similar)

30-mL Universal containers (Sterilin 128A or similar).

plastic pipettes, singly wrapped, disposable, graduated (2-, 5- and 10-mL; Sterilin or similar)

IV:C. Gametocyte Culture Protocol: Membrane Feeding Gametocytes to Mosquitoes

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1. Gametocyte culture protocol

Erythrocytes: Human O+ erythrocytes from banked blood (collected in CPD-adenine), as fresh as possible (not more than 7 days after the bleed).

Wash the red cells three times (or more if necessary) with incomplete MCM, removing any buffy coat, and resuspend with an equal volume of complete MCM. This 50% hematocrit blood should be stored at 4 ^{o}C.

Incomplete malaria culture medium (IMCM) as for the asexual cultures, but with the addition of 50 mg/liter hypoxanthine (Sigma) and the omission of $NaHCO_3$. Adjust the pH to 7.2.

Complete malaria culture medium (CMCM): Add 10 mLof human blood type O+ serum (from a blood bank, pooled from several donors, heat inactivated at 56^{o}C for 1 hour, and stored in aliquots at –70^{o}C) and 4.2 mLof a sterile, freshly made 5% solution of $NaHCO_3$ to 100 mL IMCM, made as incomplete medium not more than 2-3 weeks ago. Incubate at 37 ^{o}C overnight to check for contamination before use. Use within one week and discard if pH increases to >7.6 (pink in color).

A gametocyte-producing stock culture between 4 and 10%: Thaw fresh stock parasites every 2-3 months because the gametocyte-producing ability (and infectiousness of gametocytes) drops after a time.

The culture *must* be in good health and growing quickly. Ideally, it will need diluting down 2-3 times a week. Do not dilute cultures below 1% as this can stop them from growing well for a few days, and do not let the culture get too high (>6-8%) before you dilute it down, as it may ‘crash’. The presence of gametocytes in a stock culture is a good thing for gametocyte culture!

Method

Set up cultures in tissue culture flasks

- Small culture flasks of 25 cm^2 have an initial volume of 5 mLwhich will increase to 7.5 mL; 75-cm^2 flasks have an initial volume of 15 mLwhich will increase to 25 mL.
- Set up cultures at 0.5-0.7% parasitemia, 6% hematocrit in complete MCM. If the culture is mainly rings, set it up at 0.7%; if mainly schizonts, at 0.5%; if mixed, at 0.6%.
- Prewarm the flasks and complete MCM to 37 ^{o}C before use.
- Gas the flasks with a mixture of 1% O_2, 3% CO_2, balance N_2 (candle jars or CO_2 incubators are not recommended) for a minimum of 10 seconds (25-cm^2 flasks) or 20 seconds (75-cm^2 flasks) at a pressure of around 5 lb/in^2.

- Place in a 37 ^{o}C incubator with the flasks lying down. Replace complete MCM daily and agitate cultures gently after gassing.

It is very important to keep the cultures as warm as possible at all times. When removing the spent medium, do not take off any of the black pigment that comes off just before you start to take off blood.

Bulking up the gametocyte culture:

Once the culture has reached a high parasitemia it is 'bulked up'. This means that the volume of medium added is increased to 7.5 mL(or 25 mL) without the addition of more blood cells.

- Make thin blood smears on the fourth day after setting up the culture. Fix and stain with Giemsa's stain as previously described.
- Examine under 100x oil objective. You should look for a high parasitemia and 'stressed looking' parasites such as triangular ring stages and hazy or faint-looking trophozoites and schizonts.
- If these are not present, do not bulk up. Make blood smears on the following days and bulk up when appropriate (usually 4 or 5 days after set-up).
- As soon as the culture is judged ready, bulk up by adding extra medium to each flask to give a final volume of 25 mL(75-cm^2 flasks) or 7.5 mL(25-cm^2 flasks).
- Thereafter maintain the cultures on the increased volume of medium, changing daily and gassing.
- Prepare smears once or twice a week to check the state of your cultures and to look out for contamination.
- Gametocytes are mature from 14-17 days after the start of culture.

References

Carter R, Ranford-Cartwright LC, and Alano P. 1993. The culture and preparation of gametocytes of *Plasmodium falciparum* for immunochemical, molecular, and mosquito infectivity studies. *In:* Hyde JE, ed. Methods in molecular biology, vol. 21: Protocols in molecular parasitology. pp. 67-89. Totowa, New Jersey: Humana Press; pp. 67-89.

Ifediba T and Vanderberg JP. 1981. Complete *in vitro* maturation of *Plasmodium falciparum* gametocytes. Nature (London) 294, 364-366.

Trager W and Jensen JB. 1976. Human malaria parasites in continuous culture. Science 193, 673-675.

Haynes JD, Diggs CL, Hines FA, and Desjardins RE. 1976. Culture of human malaria parasites *Plasmodium falciparum*. Nature (London) 263, 767-769.

2. Membrane feeding gametocytes to mosquitoes

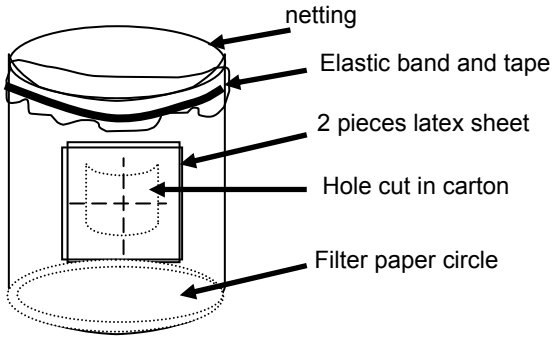
This protocol is for infection of *Anopheles* mosquitoes with *P. falciparum* gametocytes, grown according to the gametocyte culture protocol. Gametocytes should NOT be grown with gentamycin, as this can have adverse effects on the infection rates.

Note: *P. falciparum* is classified as a category 3 pathogen in some countries, which means that there are regulations regarding its safe use. Familiarize yourself with any relevant local or national safety regulations in your country before beginning work, and take steps to minimize the risk of accidental infection. This is particularly important when mosquitoes are being infected. It is necessary to have an approved secure insectary and to take every step to ensure that potentially infected mosquitoes do not escape.

Materials

14 - 17 day old gametocyte cultures
Human blood type O+ serum (from a blood bank, pooled from several donors, heat inactivated at 56 ^{o}C for 1 hour)
Human O+ erythrocytes from banked blood (collected in CPD-adenine), washed to remove the anticoagulant before use, as fresh as possible (no more than a few days since collection)
Glass membrane feeders and plastic tubing to connect them
Clamps and stands to which to attach the feeders
Circulating waterbath at 37 ^{o}C
Baudruche membrane or Parafilm sealer and small elastic bands
1-pint waxed-paper cartons (e.g., ice-cream containers), filter paper circles to fit in the bottom of the cartons, latex sheeting, nylon netting, tape.
Mosquito aspirator ('pooter')
Anopheles mosquitoes, 3-5 days postemergence
5% glucose in 0.05% PABA (4-amino benzoic acid) solution
Absorbent cotton wool

Preparation for a membrane feed



- Prepare sufficient mosquito pots for the experiments you are planning. Cut a small hole (approximately 2 cm square) in the side of a waxed paper carton. Cover this on the outside of the carton with two squares of latex sheeting (e.g. dental dam) with a slit cut into each one, and tape securely over the hole to form the leak-proof

inlet/outlet port. Fix a filter paper circle to the base of the pot. Stretch netting over the top of the pot and secure with an elastic band and tape.

- Collect female mosquitoes two days before you are going to carry out the feed itself. These mosquitoes should be between 3 and 5 days postemergence on this day (so they will be between 5 and 7 days post emergence on the day of feed).
- Place a small piece of cotton wool soaked in distilled water on top of the netting. This should be replaced daily until the day of feed.
- Attach baudruche membrane or finely stretched Parafilm to the glass feeders with an elastic band. If using baudruche it is easier to apply it wet.
- Connect the feeders to a circulating waterbath set at 37 ^{o}C, and clamp at the correct level for the mosquito pots.

Preparing the infectious feed

Work swiftly and take steps to prevent the temperature of the parasite material from dropping below 37 ^{o}C.

- Prewarm washed uninfected fresh blood and serum to 37 ^{o}C.
- Resuspend the washed uninfected fresh blood in the serum to give a final packed cell volume (pcv) of 40%. Keep warm at 37 ^{o}C.
- Remove medium from culture flasks and resuspend the cells by gentle shaking. Transfer to tubes and centrifuge to pellet (5 minutes at 1800 x *g* at 37 ^{o}C).
- Remove supernatant, measure the volume of the pellet (approximately), and add an equal volume of serum to the pellet. Mix the pellet and serum very well (remember the gametocytes stick to the side of the tube).
- Dilute the parasite/serum mix with 3 to 9 times its volume of the fresh washed uninfected fresh blood/serum.
- Mix with a Pasteur pipette and place approximately 1 mL(depends on membrane feeder size) into each feeder.
- Place the mosquito pot underneath and allow to feed for approximately 20 minutes.
- Remove the unfed mosquitoes 2-3 hours after the feed and kill by freezing at –20 ^{o}C.

To check for exflagellation

- Place a small drop of the prepared parasite/blood/serum mixture on a microscope slide, breathe on a coverslip, and place over the drop of blood. Examine microscopically (phase or Nomarski 40x objective are best) as soon as possible (always within 10 minutes).

Maintenance and dissection

- Keep infected mosquitoes in a secure insectary at 26 ^{o}C/80% relative humidity.
- Place clean cotton wool pads soaked in 5% glucose/0.05% PABA solution on top of the netting; replace these daily.
- Kill mosquitoes with chloroform, and then dip briefly into ethanol. Dissect in 1x PBS.
- Oocysts will be visible from 7 days postfeed, but are easiest to see 9-10 days postfeed (depending on the temperature of the insectary).
- Sporozoites may be seen in the salivary glands from day 14 postfeed onwards.

References

This protocol is summarized from the reference below. Please refer to this chapter for original references.

Carter R, Ranford-Cartwright LC, and Alan P. 1993. The culture and preparation of gametocytes of *Plasmodium falciparum* for immunochemical, molecular, and mosquito infectivity studies. *In:* Hyde, JE, ed. Methods in molecular biology, Vol. 21: Protocols in molecular parasitology. Totowa, New Jersey: Humana Press; pp. 67-89.

IV:D. Production of *Plasmodium falciparum* oocysts and sporozoites

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The production of different sexual stages of *P. falciparum* and their isolation from infected mosquitoes facilitates a variety of cellular, molecular, immunochemical, and transmission-blocking studies that examine the parasite-vector relationship. The procedures of mosquito rearing, parasite cultivation, and infection and dissection of mosquitoes are highly specialized skills requiring considerable experience to perform with competence. Additionally, the timing of the two life cycles, that of the parasite and that of the mosquito, requires precise planning so that the mosquitoes are the correct age to infect during the brief period in which the parasites are viable for infection.

Mosquitoes capable of transmitting *P. falciparum* should be maintained within a limited access, dedicated, high security humidified insectary. It is recommended that all rooms be painted in a light color and all cages be constructed of white materials in order to make highly visible any mosquito that may temporarily escape. A nonresidual insecticide spray should also be available in the event of an emergency. Infected mosquitoes to be dissected are first stunned or anesthetized by chloroform and then rendered incapable of movement (dewinged and delegged). Any potentially infective mosquitoes that are not to be dissected are frozen by placing the complete container at −20 °C prior to autoclaving and disposal.

Equipment required for blood feed of mosquitoes

(A) Pooter—A pooter is a device used to transfer mosquitoes by aspiration. We use a polycarbonate plastic-tubed pooter, but any toughened material is suitable provided that it is transparent. Ours are custom-made (contact details upon request) but an efficient pooter can equally well be modified from a 10-mL plastic pipette. This is prepared for use as follows:

- Insert a stainless steel (or nylon) fine-meshed disc of the internal dimensions of the pooter tube 2 cm from one end of a 20- to 25-cm tube. A ring may be etched in the internal surface of the tube in which the disc can sit securely.
- Attach a length of clear, transparent silicon rubber tubing (approximately 30 cm) over the meshed end of the polycarbonate tube.
- Use the pooter by placing the free end of the silicon rubber tubing in your mouth and aspirating by a ‘suck-blow’ technique. Transfer the mosquitoes by sucking them into the open end of the polycarbonate tube and then blowing them out into the container.

(B) Mosquito container—The ideal container should minimize the risk of mosquito escape or injury or of the handler being bitten. The container used successfully by ourselves and several other research groups is customized from a 500-mL cylindrical ice-cream container made of waxed cardboard (of the type used by Ben & Jerry´s and Häagen-Dazs). This is prepared for use as follows:

- Cut out a circular piece of filter paper (Whatman no. 1, or similar) to the internal dimensions of the container and tape it to the floor of the container to soak up any spillage that may occur (from nutrient feed, diuresis drops, etc.) when in use.

- Cut a 3-cm square in the side and cover the hole with 2 layers of dental latex (HCM rubber dental dam or similar, available from your local dental supplies distributor) into which two 1-cm cuts are made, one of which is then placed perpendicular to the other. Hold both squares of latex firmly in place with electrician's insulating tape. This results in a cross-shaped entry port through which, with the aid of a pooter, mosquitoes can be safely introduced or removed.
- To complete the container, secure a double layer of 'bridal veil' netting over the mouth of the container with elastic bands and insulating tape.
- Other than during the actual blood feed, keep the containers inside a gauze-covered cage that acts as a secondary security barrier.

(C) Blood feeder—A glass membrane feeder is used to deliver the infectious blood meal to the mosquitoes. Blood feeders consist of a water-jacketed chamber into which the infectious blood feed can be introduced. Ours are custom-made by a professional glass blower (contact details upon request). This is prepared for feeding use as follows:

- Cover the blood feed chamber with a Baudrouch membrane derived from bovine intestine (Joseph Long Inc.). Wet the membrane with tap water to ensure that, when dry, it is taut. This will encourage the mosquitoes to feed. If washed and dried thoroughly after each use, a membrane can be used 5 or 6 times before it loses its integrity. In fact, in our experience, previously used membranes are preferred to new membranes because their use results in a greater proportion of mosquitoes taking a blood meal.
- Hold the membrane in place with an elastic band.
- Pass tap water at 38 °C through the water jacket via a recirculating pump (water inlet via the lower tube, outlet via the higher tube).

Rearing of *Anopheles stephensi* mosquitoes

- Keep each stock colony of *An. stephensi* in a gauze-covered, plastic-covered wire-framed cage. We use cages of frame dimensions 30 cm^3, which are covered with mosquito netting or 'bridal veil' netting to incorporate a 15-cm front sleeve.
- Keep cages at 26.0 ± 0.5 °C and 80.0 ± 2.0% relative humidity. Maintain all colonies on a reverse light cycle with 12 h artificial lighting from 2000 to 0800 h. This enables all manipulations to be performed in keeping with the nocturnal activity of the mosquito during normal working hours.
- Feed adult female mosquitoes twice a week on uninfected rodent blood. We routinely feed directly by bite of naive outbred mice, but rats or guinea pigs are also suitable. Anesthetize the mouse by intraperitoneal injection of 0.1 mL per 10 g body weight of 10% Hypnorm (Janssen Pharmaceutica) and 20% Valium (Roche) in sterile distilled water. Place the mouse abdomen-down, with the four limbs stretched out, on top of the cage in which the mosquitoes to be fed are kept, and allow feeding for 20 min. The abdomen may be shaved of fur to facilitate access for feeding. We use mice under terminal anesthesia in accordance with project and personal licences issued by the UK Home Office and following institutional guidelines. Your local regulatory authority may permit rodents to be reused for the purposes of mosquito feeding, in which case they can be allowed to recover from the anesthetic.
- Eggs are laid by fed female mosquitoes 2 to 3 d after a blood feed. Place a small bowl lined with filter paper (Whatman no. 1, or similar) and filled with tap water on the floor of the stock cage overnight. The following morning, remove the bowl to

another cage, and cover it with a square of transparent Perspex until the eggs begin to hatch (within 2 d).

- Rear the larvae in clean plastic washing-up bowls (preferably colored white) containing 3 inches of water. Carefully transfer the contents of the hatching bowl into a larval rearing bowl (approximately 300 larvae per bowl) and discard the filter paper. The water must not contain large amounts of chlorine so it may be necessary to allow your local tap water to stand for a minimum of 24 h before use to allow any chlorine to dissipate ('chlorine-free' water). We recommend the continuous maintenance of a reservoir of chlorine-free water ready for use. Alternatively, water can be boiled before use or bottled water can be used. Whatever the source and treatment of the water, it must be at room temperature before eggs are added.
- Anopheline mosquito larvae are primarily surface feeders and must be provided with a form of food that floats on water. We use koi carp pellets (Wardley Corporation premium koi staple, or a suitable alternative supplied by your local aquarist). Baby weaning formulation milk powder ('Farex' or similar) may also be used but should to be finely sieved before sprinkling on the water.
- Feed the larvae morning and evening with fresh pellets (a 'little but often' regime). Remove and discard any surplus 'old' food. In this way, the larvae receive sufficient food, but not so much that the bowl becomes contaminated with uneaten food.
- After 2 to 3 d, reduce the mosquito numbers. By keeping the larvae numbers between 200 and 250 per bowl, large well-nourished mosquitoes are produced.
- Larvae should pupate 6 to 8 d post emergence from eggs if kept at 26 °C and 80% relative humidity. Pupation can be delayed by 2 or 3 d by reducing the ambient temperature by 1 or 2 °C. Kill surplus larvae by immersion in hot water. Do not discard them live down the sink.
- Collect pupae and place them in a bowl of chlorine-free water. Cut an inch off of the point of a filter-paper cone and place the cone over the bowl. This system allows emergence of adult mosquitoes (2 to 3 d postpupation), but also deters emergent mosquitoes from flying back into the bowl and drowning and keeps fed females from laying eggs in the pupae bowl. Place the pupae bowl in the stock cage.
- Provide a solution of 5% glucose (Sigma) and 0.05% *p*-aminobenzoic acid (PABA; Sigma) in chlorine-free water for all adult mosquitoes. Filter-sterilize this nutrient solution through a 0.22-µm filter (Sterilin or similar) before soaking a cotton wool pad and placing it in the cage. The pad should be moist but not dripping as this leads rapidly to fungal contamination, and should be changed daily.

<u>On the day before the feed</u>

- Prepare the mosquito container(s) and place into each, with the aid of a pooter, 4 to 6-d-old virgin female mosquitoes (i.e., they should be 5 to 7 d postemergence from pupae on the day of the feed). Add a suitable number of mosquitoes for the volume of the container to be used; for a 500-mL cylindrical container, we add between 100 and 250 mosquitoes. Up to 20 mosquitoes can be accommodated comfortably within a pooter at any one time to enable counting during the transfer procedure. It is important to handle the mosquitoes carefully so as not to damage them.

- Test mosquitoes for their readiness for a blood feed by evaluating their attempt to feed on a warmed, water-filled, tissue culture flask placed on top of the gauze-covered stock cage in which they are housed. If the pooter is used for transfer to the feed container while the culture flask is in place, those mosquitoes that are attracted by the warm object can be positively selected. This procedure acts not only to select older individuals more eager for blood among a population of slightly mixed ages (due to variation in the timing of emergence from pupation) but also to segregate female from male mosquitoes. Only females are attracted by the warmed flask, as its temperature mimics that of a human (from which they require blood in order to lay eggs); male mosquitoes do not blood feed and are ambivalent to the presence of the flask.
- Feed mosquitoes on a cotton wool pad soaked in 5% glucose/0.05% PABA which is placed on top of the mosquito container. Remove the cotton wool pad 12 h before the blood feed. Starving the mosquitoes for 12 h prompts the vast majority to feed to engorgement on blood.

On the day of the feed

- Wash fresh group O, Rhesus group positive whole blood and warm it to 37 °C.
- Warm group O, Rhesus group positive serum to 37 °C.

For details of preparation of human RBC and pooled serum, refer to the Appendix of MOSQUITOES AND PARASITES, section II:B.

- Place a Baudrouch membrane on the blood feeder, wet it, and allow it to dry. Once the membrane is dry (approximately 20 min), switch on the water pump and allow the water jacket to warm up to 38 °C (approximately 60 min).
- Take *P. falciparum* to be used for the feed from 14- to 17-d-old cultures which contain mature, stage V gametocytes. As male gametocytes mature more rapidly than do females, in practice it is best to use either: a) exclusively cultures that are 17-d post-subculture, which should contain a sufficient number of gametocytes of both sexes; or b) a preparation of blood of different ages (such as a 1:1 mix of 14- and 16-d-old cultures), which will show a sex ratio bias in favor of male or female parasites, respectively. For method of cultivation, refer to “Growth of blood cultures for gametocyte production” of MOSQUITOES AND PARASITES, section II:B.

If at all possible, perform the following procedures entirely at 37 °C and as rapidly as safe handling and good microbiological practice permits in order to minimize the possibility of gametocytes commiting to activation/exflagellation before the blood meal is taken up by the mosquito.

- Transfer infective cultures to centrifuge tube(s) prewarmed to 37 °C.
- Centrifuge the cultures at 800 × *g* for 2 min in a centrifuge prewarmed to 37 °C.
- Remove and discard the supernatant.
- Measure the volume of the remaining pellet, containing packed PRBC, and add an equal volume of serum.
- Make the volume of infective blood/serum from step 8 up to that of the blood feeder to be used (1 to 2 mL) by diluting the suspension up to 3× volume with a mixture of 1:1 washed human RBC/serum. Mix gently but thoroughly. We routinely dilute blood in this manner because: a) better infection rates are achieved using blood that contains a high but submaximal gametocytemia; b)

addition of fresh blood/serum to the 17-d-old cultured PRBC promotes mosquito feeding, in terms of both extending the feeding time of individual mosquitoes (qualitative) and increasing the number that feed (quantitative).

- Rapidly transfer the diluted infective blood from step 9 into the blood feeder using a prewarmed syringe with a blunt-ended needle, ensuring that there are no air bubbles present.
- Remove a mosquito container from its outer cage. Place the container underneath the blood feeder, ensuring that the blood feeder and the netting are in intimate contact.
- Turn off any artificial lighting and allow the mosquitoes to feed undisturbed for 12 to 15 min. We recommend that the high security insectary in which the mosquitoes are held be without windows; this enables feeding to take place in the dark, mimicking feeding behavior in the wild.
- Remove the mosquito container from underneath the blood feeder, thus terminating feeding, and return it to a gauze-covered cage. Most mosquitoes that have been starved of nutrients for the previous 12 h will feed to engorgement within a few minutes. These will have a very distended, red abdomen and can be easily distinguished from mosquitoes that have not fed. If the mosquitoes have fed well, the floor of the container will be covered with red splashes of fluid passed by diuresis. Depending on the ease with which the pooter can be manipulated within the container, it may be possible at this stage to remove individual unfed mosquitoes.
- During the time the blood feed is taking place, examine any infective blood/serum mix that was not added to the membrane feeder for gametocyte activation and/or exflagellation. Exflagellation is the explosive production of male gametes of the malaria parasite, which happens in the mosquito midgut within a few minutes of a blood meal. The cytoplasm of the male gametocyte becomes agitated, followed rapidly by the sudden and vigorous protrusion and detachment of up to 8 flagellated gametes (spermatozoa) from the surface of the parasite cell body. This phenomenon also occurs spontaneously in vitro and thus may be observed in cultures of fresh infected blood under the light microscope (1000× magnification under oil immersion). It is controlled in vitro solely by the change from 37 °C to the ambient laboratory temperature, the pH rise this brings being mediated by a fall in CO_2 tension as the blood equilibrates with the atmosphere. Viewing of gametogenesis (typically between 10 and 25 min after removal of a blood culture from the 37 °C incubator) is a strong indicator that oocysts and sporozoites will be produced following a blood feed. However, in our experience, observable exflagellation is not a prerequisite for successful mosquito infectivity.

On days after the feed

- Subsequent to a blood feed, feed mosquitoes daily on a 5% glucose/0.05% PABA diet, as previously described. Delaying the start of feeding by 24 h post blood feed enriches for blood-fed mosquitoes as the vast majority of ‘die off’ mosquitoes under these conditions are those that did not blood feed and therefore were starved of nutrients for at least 36 h.
- Mosquitoes may be dissected for the presence of oocysts at 10 to 12 d postblood feed. Dissection for sporozoites may take place from 14 d postblood feed. The number of live mosquitoes in each container at the time of dissection must be verified and recorded by 2 people independently (usually by observing carefully

the pootering process). The total number of mosquitoes either dissected or undissected at the end of the experiment should tally with the records made. The protocol for dissections is described below.

Dissection of blood-fed mosquitoes

Dissection of mosquito midgut to determine the presence of oocysts

- For the detection of *P. falciparum* oocysts, dissect mosquitoes 10 to 12 d postinfective blood feed.
- Remove mosquitoes from the container via a pooter, the end of which is immediately plugged with cotton wool and sealed with masking tape to prevent escape. Mosquitoes may be collected one at a time, but up to 5 mosquitoes can be held in the cavity of the pooter without compromising handler safety.
- Strike the pooter against the palm of the hand firmly several times to knock the mosquitoes out for a sufficient period of time to allow their safe removal onto a dissection board. In order to stop each mosquito from flying, and also as an aid to dissection of the body, remove its legs and one wing with a scalpel blade.
- Place dewinged and delegged mosquitoes in a watch glass containing dissection medium (PBS + 0.0001% FCS). Using a pair of fine forceps, pick up each mosquito by its one remaining wing and dip it into 70% ethanol to ensure both sterility and killing of the mosquito.
- Remove individual mosquitoes, mount them on glass microscope slides onto which a drop of dissection medium had been previously placed, and remove the remaining wing.
- Dissect the mosquito under a binocular light microscope using a 40× objective (total magnification 400×).
- Separate the abdomen from the rest of the mosquito. Remove the gut by holding the anterior of the abdomen with one dissecting needle while at the same time making a small cut with a second needle in the tegument on each side of the seventh abdominal segment. Using the second needle, gently pull on the apex of the abdomen until the gut and Malpighian tubules are exposed.
- Sever the alimentary canal sufficiently far forward to bring away (and discard) all of the foregut except for the section immediately proximal to the midgut.
- Anchor the remainder of the gut by placing the point of one needle on the posterior section of foregut. Using the other needle, cut through the alimentary canal at the junction of the midgut and the hindgut, simultaneously severing the Malpighian tubules. Discard the hindgut and Malpighian tubules, leaving only the midgut on the slide.
- When an especially rapid dissection is required (with least chance of disruption to the oocysts), once the gut has emerged completely from the abdomen, cut off and remove the hindgut only, leaving the midgut exposed but with the foregut and esophagus still attached.
- Using a needle, pick up the dissected midgut and transfer it to a fresh slide onto which has been placed a drop of dissecting medium. Lower a coverslip gently onto the moistened midgut, which then can be viewed for the presence of oocysts. The volume of medium bathing the preparation is important as too little will cause the midgut to rupture (so releasing oocysts and thereby preventing their examination *in situ*) and too much will prevent adequate flattening of the

midgut for optimal viewing. Draw off excess medium by holding a filter paper to the edge of the slide.

- View guts by under 400× magnification with reduced light when oocysts, if present, should be easily identified as circular refractive bodies on the gut wall of the dissected mosquito.
- In our experience, the number of oocysts recovered per infected mosquito varies between 1 and >200, average 30. Mosquitoes that are not infected, as determined by a lack of oocysts, are usually those that did not blood feed and will not contain eggs. Occcasionally, an infected mosquito (with oocysts) that has no eggs may also be seen.

Dissection of mosquito salivary glands to determine the presence of sporozoites

- Consider freshly dissected salivary glands containing *P. falciparum* sporozoites as potentially infectious and handle them with appropriate caution. Always wear a Howie-style laboratory coat and latex gloves.
- Sporozoites may first be observed in the salivary glands 14 to 17 d postinfective blood feed. The junction of the mosquito head and thorax contains a pair of glands, each of which is tri-lobed (or very occasionally four-lobed).
- Repeat steps 1 through 6 as described above for oocyst dissection.
- Place a dissecting needle gently on the thorax of the mosquito, just below the region where the salivary glands lie.
- Place a second needle on the 'neck' of the mosquito (apex of head and thorax) without cutting it, then with a gentle pulling action detach the head.
- The salivary glands should now be exposed and can be detached from the head. If the salivary glands are not apparent, then press with a needle again on the thorax, when the salivary glands should emerge from the thorax and can be detached.
- Transfer the dissected glands and a small volume of dissection medium into a 0.5-mL microcentrifuge tube using a 100- to 200-µL Gilson pipette. Keep the microcentrifuge tube on ice.

Liberation of sporozoites from salivary glands

- Cover the microcentrifuge tube containing the salivary glands with Parafilm to reduce the chance of leakage. Release the sporozoites by gently rupturing the glands using a laboratory benchtop whirlimixer set to maximum for 3 min or by vigorous pipetting using a 200-µL Gilson pipette.
- Once liberated from the salivary glands, the sporozoite yield can be estimated by counting using an hemocytometer. Place 10 µL of the sporozoite suspension into the hemocytometer chamber and then using 200 to 400× magnification phase-contrast microscopy to visualize the sporozoites, count the number present in a 4 × 4 grid: this number relates to the number of sporozoites per $mm^2 \times 10^4$.
- If a qualitative examination for sporozoites is all that is required, transfer salivary glands directly to a glass microscope slide. Lower a coverslip carefully onto the moistened glands and tap it gently to disrupt the glands and release any sporozoites present. These should be easily identified as comma-shaped, motile bodies.
- In our experience, the number of sporozoites recovered per infected mosquito varies between 4,000 and 30,000, average 15,000.

References
Taylor-Robinson AW, Looker M. 1998. Modulatory effect of nitric oxide on *Plasmodium falciparum* male gametocyte exflagellation. *In:* Moncada S, Toda N, Maeda H, Higgs EA, eds. The biology of nitric oxide, part 6. London: Portland Press; p. 248.
Taylor-Robinson AW, Looker M. 2000. Nitric oxide-mediated modulation of *Plasmodium falciparum* infectivity for the mosquito vector of malaria. *In:* Moncada S, Gustafsson LE, Wiklund NP, Higgs EA, eds. The biology of nitric oxide, part 7. London: Portland Press; p. 184.

IV:E. Cultivation of *Plasmodium berghei* ookinetes with insect cells for production of young oocysts

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In the method described, *Plasmodium berghei* ookinetes invade co-cultured *Aedes aegypti* Mos 20 cells and form intracellular oocysts, expressing CSP after 6-7 days; development does not appear to continue further. Other insect cell lines tested also sustain oocyst development, such as *Drosophila melanogaster* S2 cells and *Anopheles gambiae* Sua5B cells. When using other cell lines the method should be adjusted for the optimal growth of the cells used.

Equipment

haemocytometer
LabTek 8 well chamber slides (Nunc) or multiwell plates
incubator at 19 ^{o}C.
epifluorescence or confocal microscope

Materials and reagents

In vitro cultured ookinetes enriched by the magnetic bead method (this book) or using any of the methods described in references 1-3.
Insect cell line *Aedes agypti* Mos20 (ATCC # CCL-125) (see Appendix)
M199 insect cell culture medium (see Appendix)
PBS
fixative for example: aceton/methanol 1:1 or 4% paraformaldehyde, 0.2 % triton in PBS
normal goat serum (NGS) or bovine serum albumin (BSA)
suitable primary antibodies
secondary antibodies conjugated to a suitable dye for detection; for example Alexa-conjugated secondary antibodies (available from Invitrogen)

Procedure

Setting up the co-cultures

- Determine the concentration of the purified ookinetes by counting in a haemocytometer. Note that the ookinetes are often seen in aggregates after purification, and one has to count the ookinete in the aggregates as well. Diluting the ookinetes to lower cell density (<10^6/mL) in PBS and leaving them for 15 minutes at room temperature results in looser aggregates, which are easier to count.
- Determine the concentration of the Mos20 cells in a haemocytometer.
- Mix 1x10^4 ookinetes in PBS (should not correspond to more than 10 µL) and 2x10^5 cells in a total of 0.4 mLof medium M199. Add to the culture dish. Incubate at 19 ^{o}C for the requested number of days.
- The culture medium should be changed twice a week during prolonged incubations.

Monitoring the co-cultures

The co-cultures can be monitored in a number of ways.

- If the parasite is expressing GFP (green fluorescent protein) the culture can be monitored directly under a fluorescence microscope.
- Oocyst development can be detected by staining the culture with antibodies. Young oocysts will express the P28 surface antigen, and at day 6 -7 the CSP (circumsporozoite protein) antigen.
- Nuclear division can be detected using a suitable DNA binding dye, e.g. DAPI, Propidium iodide or Hoechst 33 342 .

Antibody staining of co-cultures (from LabTek chamber slides)

- Carefully remove the medium from the well. The Mos20 cells and ookinetes/oocysts stick to the plastic.
- Leave the dish at room temperature to dry. Warming the slide on a slide warmer at ~50 ^{o}C for about 10 min. improves morphology of the sample.
- All the following steps are carried out at room temperature.
- Fix the sample in suitable fixative. We have had good results using the following two protocols but each antibody should be tested for the best fixation method.
  Aceton/methanol 1:1, 2 min or
  4% paraformaldehyde, 0.2 % triton in PBS for 10 min
- After fixation rinse twice with PBS.
- Add the primary antibody diluted in PBS with 5 % NGS or 0.1 % BSA. Incubate for 30 min. at room temperature.
- Wash twice with PBS for five min. each.
- Add the secondary antibody diluted in PBS with 5% NGS or 0.1 % BSA. Incubate 30 min. at room temperature.
- Wash twice with PBS for five min. each.
- In addition, the sample can be incubated with a suitable DNA stain diluted accordingly.
- Incubate for 5-15 min.
- Wash twice with PBS.
- Mount the sample and analyze in an epifluorescence or confocal microscope.

Appendix. Growth of *Aedes aegypti* Mos20 cells.

The cells are grown in tissue culture flasks in M199 medium (Gibco) supplemented with 10% foetal calf serum and penicillin/streptomycin 50ug/mL/50µg/mL. The flasks are incubated in a stationary incubator at 25 ^{o}C. No gassing is necessary. Medium should be changed twice a week and the flasks should be split once a week.

References

Sinden RE. 1997. Infection of mosquitoes with rodent malaria. In: Crampton JM, C.B. Beard, C. Louis, editor. The molecular biology of insect disease vectors A methods manual. London: Chapman and Hall. p 67-91.

Rodriguez MC, Margos G, Compton H, Ku M, Lanz H, Rodriguez MH, Sinden RE. 2002. *Plasmodium berghei*: routine production of pure gametocytes, extracellular gametes, zygotes, and ookinetes. Exp Parasitol 101(1):73-6.

Sinden RE, Butcher, G.A., Beetsma, A. 2002. Malaria Methods and Protocols. In: Doolan DL, editor. Methods in Molecular Medicine. Totowa, New Jersey: Humana Press. p 25-40.

Siden-Kiamos I, Vlachou D, Margos G, Beetsma A, Waters AP, Sinden RE, Louis C. 2000. Distinct roles for Pbs21 and Pbs25 in the *in vitro* ookinete to oocyst transformation of *Plasmodium berghei.* J Cell Sci 113 Pt 19:3419-26.
Singh KR. 1971. Propagation of arboviruses in Singh's *Aedes* cell lines. I. Growth of arboviruses in *Aedes albopictus* and *Aedes aegypt*i cell lines. Curr. Top. Microbiol. Immunol. 55:127.

IV:F. Enrichment of *Plasmodium berghei* ookinetes from *in vitro* cultures using Dynabeads

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The method, using magnetic beads coated with a monoclonal antibody against the Pbs21 surface antigen, provides a rapid protocol for purification of small scale *in vitro* cultures of *P. berghei* ookinetes (originating from 1 to ~5 mice). Because the antigen is also present on female gametes and zygotes the purified material will contain also these stages.

Materials and reagents

in vitro cultures of *Plasmodium berghei* ookinetes, prepared as described in references 1-3.
Dynabeads® M-450 Goat anti-Mouse IgG (Invitrogen; cat. no. 110.05)
13.1 monoclonal antibody (can be obtained from Prof. R. E. Sinden, Division of Cell and Molecular Biology, Imperial College London, Sir Alexander Fleming Building, Imperial College Road, London SW7 2AZ, United Kingdom)
sterile PBS (per litre)
NaCl 8.0 g
KCl 0.2 g
Na_2HPO_4 1.15 g
KH_2PO_4 0.2 g/litre
pH=7.2

Equipment

Magnetic Particle Concentrator, MPC, (for example Dynal MPC™-S Magnetic Particle Concentrator from Invitrogen, cat. no. 120.20D)
table top centrifuge
tubes suitable for the MPC
centrifuge tubes
microscope
haemocytometer
rotating wheel

Procedure

Preparation of magnetic beads coated with 13.1 antibody

- Re-suspend the magnetic beads in their vial by mixing with the use of a vortex.
- Remove 200 µL to a suitable microfuge tube. Put the tube on the MPC for 2 min.
- Remove the supernatant while keeping the tube on the MPC.
- Washing: Add 200 µL PBS, re-suspend the beads by using the vortex, then put the tube on the MPC. Remove the PBS.
- The washing step is repeated a total of four times.

- After the last wash, remove the PBS and add 100 µL of the 13.1 antibody. Gently re-suspend the beads in the antibody solution, and then incubate for 30 min. at 4 ^{o}C, on a rotating wheel.
- Separate the now coated beads from the antibody solution by putting the microfuge tube on the magnetic rack for 2 min.
- Remove the antibody to a fresh tube; the antibody can be re-used for coating several times if it is kept at 4 ^{o}C under sterile conditions.
- Wash the coated beads with PBS four times.
- Re-suspend the beads in 200 µl PBS. Keep at 4 ^{o}C until use. The beads can be kept for > 1 month.

Purification of ookinetes using magnetic beads coated with the 13.1 antibody

- Set up an *in vitro* culture of ookinetes, starting from the blood of 1-5 infected mice as described in ref. 1-3. Incubate at 19 ^{o}C for 20-24 hours.
- Check the culture under a phase contrast microscope for the presence of ookinetes.
- Spin down the cells using a table-top centrifuge at 500xg for 5 min.
- Re-suspend the pellet in 0.5 mL PBS (per 1 mLof blood added to the *in vitro* culture) and transfer to a suitable tube for the MPC.
- Add 2.5 µL of the 13.1 antibody coated beads.
- Invert the tube for 5 min, by hand or on a rotating wheel at room temperature.
- Put the tube on the MPC for 2 min. Carefully remove the supernatant. The ookinetes attached to the beads should form a thick “pellet” close to the magnet.
- Wash the ookinetes by adding 200 µL PBS while the tube is still on the MPC. Remove the PBS carefully.
- Resuspend the ookinetes in 100 µL to 1 mLof PBS.
- Dilute a small sample (1 to 5 µl) in PBS and count the ookinetes in a haemocytometer. We usually obtain $5x10^6$ to $>10^7$ per 1 mLof mouse blood added to the *in vitro* culture. Note that the ookinetes are often seen in aggregates after purification, and one has to count the ookinete in the aggregates as well. Diluting the ookinetes to lower cell density in PBS ($<10^6$/mL) and leaving them 15 minutes at room temperature results in looser aggregates, which are easier to count.
- The ookinetes can be kept in solution for several hours at room temperature without loss of viability.

References

Sinden RE. 1997. Infection of mosquitoes with rodent malaria. In: Crampton JM, C.B. Beard, C. Louis, editor. The molecular biology of insect disease vectors A methods manual. London: Chapman and Hall. p 67-91.

Rodriguez MC, Margos G, Compton H, Ku M, Lanz H, Rodriguez MH, Sinden RE. 2002. *Plasmodium berghei*: routine production of pure gametocytes, extracellular gametes, zygotes, and ookinetes. Exp Parasitol 101(1):73-6.

Sinden RE, Butcher, G.A., Beetsma, A. 2002. Malaria Methods and Protocols. In: Doolan DL, editor. Methods in Molecular Medicine. Totowa, New Jersey: Humana Press. p 25-40.

Siden-Kiamos I, Vlachou D, Margos G, Beetsma A, Waters AP, Sinden RE, Louis C. 2000. Distinct roles for Pbs21 and Pbs25 in the *in vitro* ookinete to oocyst transformation of *Plasmodium berghei*. J Cell Sci 113 Pt 19:3419-26.

IV:G. Motility assays of *Plasmodium berghei* ookinetes

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Equipment

haemocytometer
LabTek 8 well chamber slides (Nunc) or multiwell plates.
incubator at 19 ^{o}C.
microscope equipped with a camera for time-lapse video microscopy (for Method 1) or fluorescence microscope (for Method 2)
slides and coverslips
Cytochalasin D

Materials and reagents

In vitro cultured ookinetes enriched by the magnetic bead method (this book) or using any of the methods described in reference 1-3.
insect cell line *Aedes agypti* Mos 20 (ATCC # CCL-125) (see Appendix)
M199 insect cell culture medium (see Appendix)
PBS
fixative aceton/methanol 1:1
normal goat serum (NGS) or bovine serum albumin (BSA)
anti-Pb70 antibody against an ookinete cytoskeletal protein (can be obtained from Prof. R.E. Sinden Division of Cell and Molecular Biology, Imperial College London, Sir Alexander Fleming Building, Imperial College Road, London SW7 2AZ, United Kingdom)
secondary antibodies conjugated to a suitable dye for detection; for example Alexa conjugated secondary antibodies available from Invitrogen
Vaseline

Method 1. Direct observation of motile ookinetes.

- Resuspend the purified ookinetes to a final concentration of ~ $5x10^6$ – 10^7 ookinetes/mL.
- Mix 1 µL the purified ookinetes with $2x10^5$ freshly harvested *Aedes aegytpi* Mos20 cells in 10 µL M199 medium.
- Add the cell mixture to a glass slide and cap the slide with a Vaseline rimmed cover slip.
- The slides should be observed immediately as motility is rapidly initiated. Motility of the ookinetes is monitored at room temperature, under a microscope using transmission optics. For time-lapse photography pictures are taken every 30 seconds. Alternatively, when using GFP expressing parasites a fluorescence microscope can be used. The processing of the time-lapse videos is easier using the fluorescent parasites as the insect cells will not be visible under these conditions.

Method 2. A quantitative assay of motility.

Setting up the co-cultures

- Mix $1x10^4$ ookinetes in PBS (should not correspond to more than 10 µL) and $2x10^5$ *Aedes aegypti* Mos20 cells in a total of 0.4 mLof medium M199.

- Add the cell mixture to the culture dish (8 well Lab Tek chamber slides or 24 well multiwell plate). In each experiment a control with Cytochalasin D at a final concentration of 10 µM should be included.
- Incubate at 19 ^{o}C over night.

Antibody staining of co-cultures (from LabTek chamber slides)

- Carefully remove the medium from the well.
- Leave the dish at room temperature to dry. Warming the slide on a slide warmer at ~50 ^{o}C for about 10 min. improves morphology of the sample.
- Fix with aceton/methanol 1:1, for 2 min at room temperature.
- After fixation rinse twice with PBS.
- Add the anti-Pb70 antibody diluted in PBS with 5 % normal goat serum or 0.1% BSA. Incubate for 30 min. at room temperature.
- Wash twice with PBS for five min. each.
- Add the secondary antibody diluted in PBS with 5% NGS or 0.1 % BSA. Incubate for 30 min. at room temperature.
- Wash twice with PBS for five min. each.
- Mount the sample.

Scoring the samples

- Score the stained samples under the fluorescence microscope:
    - In the control sample with wild type ookinetes most parasites should be seen as clearly demarcated, individual ookinetes.
    - In the control sample with Cytochalasin D most ookinetes should be seen in aggregates. Cytochalasin D inhibits motility of the ookinete and therefore the numbers from these samples represent the aggregated non-motile ookinetes, while the single ookinetes results from scattering of the ookinetes when the cultures are seeded.
- To achieve a quantitative estimate of motility count individual and aggregated ookinetes in random microscope fields.

Appendix. Growth of *Aedes aegypti* Mos20 cells.

- The cells are grown in tissue culture flasks in M199 medium (Gibco) supplemented with 10% foetal calf serum and penicllin/streptomycin 50ug/mL/50µg/mL.
- The flasks are incubated in a 25 ^{o}C stationary incubator.
- No gassing is necessary.
- Medium should be changed twice a week and the flasks split once a week.

References

Sinden RE. 1997. Infection of mosquitoes with rodent malaria. In: Crampton JM, C.B. Beard, C. Louis, editor. The molecular biology of insect disease vectors A methods manual. London: Chapman and Hall. p 67-91.

Sinden RE, Butcher, G.A., Beetsma, A. 2002. Malaria Methods and Protocols. In: Doolan DL, editor. Methods in Molecular Medicine. Totowa, New Jersey: Humana Press. p 25-40.

Rodriguez MC, Margos G, Compton H, Ku M, Lanz H, Rodriguez MH, Sinden RE. 2002. *Plasmodium berghei*: routine production of pure gametocytes, extracellular gametes, zygotes, and ookinetes. Exp Parasitol 101(1):73-6.

Siden-Kiamos I, Ecker A, Nyback S, Louis C, Sinden RE, Billker O. 2006. *Plasmodium berghei* calcium-dependent protein kinase 3 is required for ookinete gliding motility and mosquito midgut invasion. Mol Microbiol 60(6):1355-63.
Siden-Kiamos I, Pinder JC, Louis C. 2006. Involvement of actin and myosins in *Plasmodium berghei* ookinete motility. Mol Biochem Parasitol 150(2):308-17.

V. Transmission blocking and sporozoite invasion assay

V:A. Transmission blocking assay (TBA) – membrane feeding

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Equipment

- membrane feeder (glass), Lillie Glassblower, USA (Tel: + 1-404-436-8959)
- silicon tubing to connect the feeders
- aspirator tube
- pint containers (Naptune Brand Round containers, Kahn Paper Co.)
- dental dam, hygienic
- nylon net, bridal net (any fabric store).
- Parafilm
- water pump, constant temperature circulator (Polystat, Cole-Parmer, or any other equivalent)
- light microscope (e.g., Olympus)
- dissecting microscope (e.g., Olympus)
- dissecting forceps
- dissecting glass slide
- glass slides and coverslips
- Eppendorf tubes
- sterile pipettes
- insectaries approved for handling *Plasmodium falciparum*-infected mosquitoes

Materials and reagents

- parasite culture:
  Maintain *Plasmodium falciparum* gametocyte culture for 14 to 18 days making blood smears on alternate days. From *Day 12* onward, make blood smears on a daily basis and look for mature C-shaped gametocytes as well as in vitro exflagellation of male gametocytes.
- mosquitoes:
  Maintain and breed *Anopheles stephensi* according to the standard procedure (see MOSQUITOES AND PARASITES, section II:C). Use 3- to 5-d-old mosquitoes for the experiment.
- human RBC

heat-inactivated normal human serum (NHS)
heat-inactivated antiserum
incomplete medium used for *P. falciparum* cultures
mercurochrome solution

Methods

Important notes before starting

- *Plasmodium falciparum* cultures of at least 0.5% mature gametocytes and at least 1 to 2 exflagellation centers per field should be used for mosquito feeding.
- Before preparing blood meal, have mosquitoes and feeders ready.
- Take care to maintain the parasite and the blood always at 37 °C.

Exflagellation assay

- Using a sterile pipette, take out 1 mL of parasite culture and transfer it to an Eppendorf tube.
- Spin down the tube for 15 s at high speed and discard the supernatant.
- Add an equal volume of normal human serum to resuspend the pellet and allow it to stand for 10 min at room temperature.
- Place 10 μL of this suspension on a glass slide and cover it with a coverslip.
- Determine the average number of exflagellation centers per field using a light microscope at 400× magnification. A polarizer filter may help in locating the parasitized RBC as in this configuration the malaria pigments look shiny. Scan at least 10 to 20 fields to get a proper estimate of exflagellation centers.

Making dilution of antibodies in 50% RBC

Premix the antibodies to be tested for transmission blocking activities with 50% red blood cells in heat-inactivated normal human serum at various dilutions (1:5 to 1:20). Keep these diluted antibodies at 37 °C until the final feeding.

- Place 100 μL of 50% RBC (in incomplete medium) in a microfuge tube, harvest the cells at 3,000 rpm for 1 minute, and wash them with heat-inactivated NHS.
- Resuspend the cells at 50% hematocrit in NHS and antiserum to obtain desired serum dilution.
- Incubate the tubes at 37 °C.

Preparation of mosquito pint

- Make a hole (2-cm diameter) in the paper can. Cover the hole with two squares (2 in × 2 in) of dental rubber with perpendicular cuts on top of each other using adhesive tapes.
- Cover the top of the paper can with nylon net of appropriate size and securely tape the net to the paper can.

Collecting female mosquitoes in pint

Distribute 30 to 40 female mosquitoes per pint. The easiest way to collect female mosquitoes from a mixed pool of male and female is to place a warm water bottle (39 to 40 °C) against the wall of the mosquito container. The female mosquitoes are attracted to the warmer side of the cage, whereas the male mosquitoes will be all over. Starve the mosquitoes for 5 to 6 h.

Preparation of feeders

- Connect the glass feeders to each other and to the circulation water pump with silicon tubing to generate a continuous flow of 39 °C hot water through the water jackets of the membrane feeders.
- Stretch Parafilm membrane (approximately 2 in × 2 in) in both directions and place on the glass feeder. Maintain the temperature of the membrane feeder by a glass water jacket (39 °C) and a circulating water bath.
- Place the pint with female mosquitoes under the warmed membrane feeder.

Membrane feeding

This step is the most crucial and must be performed as quickly as possible. During the whole procedure, take care not to allow the temperature of the *Plasmodium falciparum* gametocyte culture to drop below 37 °C. A drop in temperature promotes exflagellation. The success of the experiment depends on the maximization of exflagellation as well as gametogenesis within the mosquito, not outside. Allow the mosquitoes to engorge for 15 min.

Experimental steps

- Place 10 mL of matured *Plasmodium falciparum* gametocyte culture in a 50-mL conical tube.
- Harvest at 1,000 rpm for 2 min and then allow the rotor to stop naturally (without the break).
- Remove the supernatant and resuspend the pellet at 50% hematocrit with prewarmed NHS.
- Keep the tube warm at 37 °C.
- Lower the membrane feeder over the nylon net covering the pint. Make sure that the mosquitoes are attracted to the membrane.
- Add 50 μL of gametocyte suspension kept warm at 37 °C to the diluted antibody and immediately transfer the mixture to the feeders.
- After 15 min, separate the unfed mosquitoes from the fed ones. It is very easy to separate the fed mosquitoes from unfed mosquitoes as the fed mosquitoes have swollen red abdomens.
- Feed sugar and water to the mosquitoes and maintain them for 7 to 9 more days in a room maintained at 26 °C and 60 to 80% relative humidity.

Scoring and statistical analysis

Perform any TBA experiment with at least 2 or 3 different concentrations of the test serum. The controls may include preimmune sera, irrelevant antisera (raised against asexual stage specific antigens), no antiserum added, and a serum known to possess transmission blocking activity.

- After 7 to 9 days, dissect the midgut of each and every individual mosquito from different groups under the dissection microscope and look for the presence of oocysts. Mosquitoes can be knocked down by dropping them in 70% ethanol and then transferred to a container with 1× PBS. Transfer the mosquitoes to glass slides and carefully pull the posterior part of mosquito with dissecting forceps, holding the anterior part of mosquito at the same time with another forceps. Treat the mosquito midguts thus separated with drops of 0.1% Mercurochrome solution and allow them to stain for a few minutes under coverslips.

- The oocysts will stain red on a light pink background of the midgut. Count the number of oocysts per midgut.
- Calculate the infection rates (number of mosquitoes infected per total number dissected) and number of oocysts (geometric means) in mosquitoes.
- Compare the infection rates and the number of oocysts in mosquitoes fed test serum to those obtained from feeds using appropriate control sera.
- Perform the Mann–Whitney test to assess the statistical significance.

Comments:

Apart from assessing the potency of different transmission blocking antibodies, the same experimental procedure can be used to address other biologically important questions. For example, effects of some chemotherapeutic drugs as transmission blocking agents can easily be scored with the help of this assay. Also the involvement of different agents on parasite infectivity can be quantitated. A similar experimental design is also used to perform genetic crosses using different parasite strains.

Reference

Bhattacharyya MK, Kumar N. 2001. Effect of xanthurenic acid on infectivity of *Plasmodium falciparum* to *Anopheles stephani*. Int J Parasitol 31(10):1129-1133.

V:B. Sporozoite invasion assay

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The techniques used for assessing *Plasmodium berghei* and *P. falciparum* sporozoite invasion are identical, although the latter parasite obviously poses a substantial risk to any individual exposed to it, so due care should be taken in handling infectious mosquitoes and viable sporozoites. Sporozoites are layered over hepatoma (HepG2-A16) cells or primary human hepatocytes and recognized by monoclonal antibody or serum raised to circumsporozoite (CS) protein, which bind the cell surface of sporozoites and block sporozoite invasion into hepatoma cells in vitro.

The reagent that is recommended for sporozoite detection is an IgM monoclonal antibody (MAb 36) which reacts with the CS proteins of both *P. falciparum* and *P. berghei*, and which was isolated from *P. falciparum* sporozoite-immunized mice (Sina et al. 1992; available on request from MR Hollingdale). In assays of biological activity, Mab 36 induces the CS precipitation reaction with live sporozoites and blocks the invasion of hepatoma cells by sporozoites in vitro at concentrations much lower than those observed for other reported CS protein-specific MAb. However, suitable alternatives are available in the MR4 repository: rabbit antiserum against *P. falciparum* CS protein (MRA-24) and rat antiserum against recombinant *P. falciparum* CS protein region I to C-terminus (MRA-21).

Equipment

- CO_2-incubator
- 200-µL Gilson pipette
- hemocytometer
- phase-contrast microscope

Materials and reagents

- immortalized human hepatocyte cell line HepG2 (available from MR4 as either a frozen ampoule or a growing culture)
- entacin–collagen–laminin (ECL)
- serum-free RPMI 1640 medium
- mosquitoes (see MOSQUITOES AND PARASITES, section II:C)
- PBS
- PBS + 0.0001% FCS
- PBS + 1% BSA
- MEM; minimal essential medium (Sigma)
- 10% heat-inactivated FCS (referred to as ‘binding medium’)
- 100% methanol
- MAb 36
- goat anti-mouse peroxidase conjugate (DAKO)
- distilled water

DAB tablet (Sigma 'Fast' DAB kit)
H_2O_2 (Sigma 'Fast' DAB kit)
DPX mountant (HD Supplies)
8-well Nunc slides

Producing a confluent HepG2 monolayer to study sporozoite invasion

The growth of HepG2 cells on 8-well Nunc coverslips is achieved using standard culture techniques, splitting confluent cultures between 1:3 and 1:6. However, the surface of slide wells should to be precoated with an extracellular matrix (entacin–collagen–laminin; ECL) to promote cell attachment.

- Dilute a 40-µL frozen aliquot of ECL (stock concentration at 1 mg/mL) in 4 mL of serum-free RPMI 1640 medium. Add 200 µL of this solution to each well of the 8-well slides.
- Leave at 37 °C for a minimum of 2 h.
- Aspirate off the ECL solution and add a 250-µL suspension of HepG2 cells to each well.
- Place slides in an incubator (37 °C, CO_2) for 2 to 3 d to allow the cells to attach and grow before using them in an invasion assay.

Invasion assay

Consider freshly dissected salivary glands containing *P. falciparum* sporozoites as potentially infectious and handle them with appropriate caution. Always wear a Howie-style laboratory coat and latex gloves.

- Dissect the mosquitoes for sporozoites between 14 and 17 d after an infectious feed, as described in detail previously (see MOSQUITOES AND PARASITES, section II:C).
- Dissect out both salivary glands from the apex of head and thorax of each mosquito and remove any unwanted body tissue. Collect salivary glands into ice-cold PBS + 0.0001% FCS. In our experience, the number of sporozoites recovered per infected mosquito varies between 4,000 and 30,000, average 15,000.
- Using a 200-µL Gilson pipette, vigorously pipette the suspension containing mosquito salivary glands up and down to disrupt the glands in order to release free sporozoites.
- Determine the density of sporozoites in this suspension using a hemocytometer. Place 10 µL of the sporozoite suspension into the hemocytometer chamber, use phase-contrast microscopy to visualize the sporozoites, and count the number present in a 4×4 grid. This number relates to the number of sporozoites per $mm^2 \times 10^4$.
- Dilute and divide the sporozoite suspension so that there are between 10,000 and 20,000 sporozoites per 300 µL of minimal essential medium (MEM) + 10% heat-inactivated FCS (referred to as 'binding medium'). Ensure that this density is the same for all groups that are to be compared.
- Remove 8-well slides containing HepG2 cells from the 37 °C CO_2 incubator, wash each well once with 300 µL of binding medium, and add 300 µL of sporozoite suspension. Place slides in a loose-lid container and return them to the 37 °C CO_2 incubator for 3 to 4 h.
- Gently aspirate off the sporozoite suspension, add 300 µL of warmed MEM (without FCS), and leave the slides for 5 min.

- Repeat the wash.
- Aspirate off the MEM and flood the wells with 100% methanol. After 5 min, remove the methanol, add PBS, and leave them at 4 °C overnight.

Visualization of sporozoites

Visualize the sporozoites using immunohistochemical techniques.

- Discard the PBS solution and remove the plastic well surrounds and the rubber gaskets from the 8-well slides.
- Block each monolayer with a 100-µL solution of PBS + 1% BSA for 10 min.
- Aspirate off the solution and add 100 µL of PBS + 1% BSA + 5 µg/mL of MAb 36 (shows immunological cross-reactivity between *P. falciparum* and *P. berghei* CS proteins).
- Incubate the slides in a humidified chamber at room temperature for 30 min.
- Aspirate off the primary antibody solution and wash the cell monolayers by placing the slides in a Coplin jar containing PBS for 2 min. Repeat this step twice more.
- Add 100 µL of PBS + 1% BSA containing a 1:200 dilution of goat anti-mouse peroxidase conjugate. Incubate the slides in a humidified chamber at room temperature for 30 min.
- Repeat the PBS washes.
- In 1 mL of distilled water, dissolve one DAB tablet and one H_2O_2 tablet from the Sigma 'Fast' DAB kit. Add 100 µL of this solution to each cell monolayer. Allow the reaction to proceed for 2 min and then terminate it by washing off the DAB solution with tap water. Note: **DAB is very toxic.** Always wear gloves when handling it and use copious amounts of water to wash solutions containing DAB down a suitable laboratory sink.
- Allow slides to dry and then mount them using DPX mountant and glass coverslips. Visualize sporozoites under either 200× or 400× magnification using phase contrast microscopy.

References

McCormick CJ, Tuckwell DS, Crisanti A, Humphries MJ, Hollingdale MR. 1999. Identification of heparin as a ligand for the A-domain of *Plasmodium falciparum* thrombospondin-related adhesion protein. Mol Biochem Parasitol 100(1):111-124.

Sina BJ, Wright C, Ballou R, Hollingdale M. 1992. A protective monoclonal antibody with dual specificity for *Plasmodium falciparum* and *Plasmodium berghei* circumsporozoite proteins. Exp Parasitol 74(4):431-440.

ANIMAL MODELS

I. Infection of monkeys with *Plasmodium* spp.

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Equipment

compound microscope
centrifuge
water bath (37 °C)
liquid N_2 freezer
Neubauer chamber
Monolet

Materials and reagents

syringes
16g, 19g, 21g, and 27g needles
25 × 75-mm microscope slides
cryovials (Nunc)
Glycerolyte 57 (Baxter Health Care - Fenwall Division)
Giemsa stain
heparinized vacutainer tubes
phosphate buffer (pH 7.0)
RPMI 1640
50-mL centrifuge tubes
12% NaCl
1.6% NaCl
0.9% NaCl + 2% dextrose solution

Preparations

Infection with fresh parasitized erythrocytes

- Collect blood from donor animal (using femoral vein) into a heparinized tube.
- Based on the parasite count, aseptically dilute blood in RPMI 1640 to give the required number of parasites in 1 mL or less.
- Restrain (New World) or anesthetize (larger Old World) monkeys.
- Inject blood slowly into femoral vein using a 25 g needle; maintain pressure on the needle exit hole until bleeding stops.

Infection with frozen parasitized erythrocytes

- Thaw vial(s) of frozen blood rapidly in 37 °C water bath.
- Rapidly transfer cells to a 50-mL tube by sterile pipette (note volume).
- Poke a hole in the cap of the 50-mL tube with a 16g needle.
- Draw 0.2× blood volume of 12% NaCl into a syringe and 21g needle.
- Add 12% NaCl dropwise through the hole while shaking the tube.
- Let the tube stand at room temp for 5 min without shaking.
- Take up 10× volume (of cells) of 1.6% NaCl.
- Add the NaCl solution dropwise through the hole while shaking the tube.

- Spin the tube at 1,400 rpm for 10 min.
- Remove the supernatant by aspiration and suspend the pellet by gentle shaking into 10× cell volume of 0.9% NaCl and 2% dextrose solution as above.
- Spin at 1,400 rpm for 10 min.
- Remove the supernatant by aspiration and suspend the pellet by gentle shaking into 1 mL of RPMI 1640.
- Inoculate recipient monkey via the femoral vein using a syringe with 25g needle.

Comment

Viable parasites have been stored frozen for >25 years; thawing and refreezing has markedly reduced their viability.

Infection via mosquito bite

- Cage infected mosquitoes in a small container covered with nylon netting.
- Anesthetize and immobilize the monkey on a restraining board using Velcro straps (the center of the restraining board is cut out to allow the belly of the monkey to rest directly on top of the feeding cage).
- Allow mosquitoes to feed to engorgement (usually 10 to 15 min).
- After feeding, dissect mosquito salivary glands and examine them for presence of sporozoites to determine number of infected mosquitoes.
- Determine the number of sporozoites remaining in the glands:
  1+ = 1 to 10 sporozoites
  2+ = 11 to 100 sporozoites
  3+ = 101 to 1,000 sporozoites
  4+ = >1,000 sporozoites
- Record the total number of +'s for all mosquitoes fed on the animal to determine the exposure index.

Infection with sporozoites harvested by dissection

- Kill mosquitoes by exposure to chloroform.
- Remove legs and wings.
- Transfer bodies to a clean slide (wiped with alcohol just prior to use).
- Remove salivary glands from the thorax by gently pulling the base of the head from the body using either a fine needle or a 27g needle on a 1-mL syringe into a drop of 20% heat-inactivated monkey serum/saline (homologous species).
- Cut the salivary duct and remove the body and head. Ten or more sets of glands can be harvested into one drop of serum/saline.
- Add a coverslip and apply gentle pressure to rupture glands.
- Gently remove the coverslip and wash sporozoites from coverslip onto the slide with several drops of serum/saline.
- Aspirate the sporozoite suspension into a syringe with 25g needle.
- Transfer the suspension to a Nunc vial.
- Adjust the volume to approximately 1 mL.
- Repeatedly aspirate and express the suspension from the syringe to further dissociate sporozoites from salivary gland tissue.
- Transfer a small volume to a Neubauer cell counting chamber and allow it to set for 15 min.
- Calculate the number of sporozoites available.
- Dilute the suspension to the desired number of sporozoites in 1 mL serum/saline.

- Inject into the femoral vein using a 25g needle.

  Comments
  Infection of New World monkeys with *P. falciparum* and *P. vivax* via sporozoites has required the injection of large numbers of sporozoites, either via the bites of many heavily infected mosquitoes or via needle-injection of dissected sporozoites. Increasing the number of sporozoites injected has resulted in greater transmission rates and shorter prepatent periods.

Monitoring parasitemia in the infected monkey

- Remove the animal from the cage and immobilize it.
- Thoroughly clean either an area of the tail or the back of the leg.
- Prick with a "Monolet", carefully avoiding the vein.
- From the drop of blood expressed, make thick and thin blood films on a precleaned, prelabeled microscope slide. Label the slide with the animal number and date.
- After the thin film dries, fix it with methanol.
- Stain the slide with Giemsa stain for 35 to 45 min.
- Examine the slide for presence of parasites.
- Express the parasite counts as
  1) parasites per 10,000 RBC,
  2) parasites per 100 WBC, or
  3) parasites per μL.
- If the latter, the method of Earle and Perez is preferred. Using a 5-μL pipette, collect the blood and express it onto a 15-mm × 5-mm area of a precleaned, prelabeled microscope slide (area is usually prescribed on the slide tray under the slide). Add a second drop of blood to the same slide to make the thin film.

Freezing parasitized erythrocytes

- Collect blood from the femoral vein into a heparinized tube.
- Spin the tube for 10 min at 1,200 rpm.
- Estimate the packed cell volume.
- Remove the plasma.
- Wash the cells 2 times with RPMI.
- Calculate 2× RBC volume (volume of Glycerolyte needed).
- Dropwise add one-fifth of the Glycerolyte volume to the tube at 1 or 2 drops per second with shaking, then let the tube stand for 5 min.
- Add the remaining Glycerolyte dropwise at same rate with shaking.
- Aliquot 0.5 mL to 1.0 mL per labeled Nunc vial.
- Transfer the vials to a −70 °C freezer overnight; transfer them to a vapor phase liquid nitrogen freezer for storage.

References

Earle WC, Perez M. 1932. Enumeration of parasites in the blood of malarial patients. J Lab Clin Med 17:1146-1168.

Sullivan JS, Strobert E, Yang C, Morris CL, Galland GG, Richardson BB, Bounngaseng A, Kendall J, McClure H, Collins WE. 2001. Adaptation of a strain of *Plasmodium vivax* from India to New World monkeys, chimpanzees, and anopheline mosquitoes. J Parasitol 87(6):1398-1403.

II. Infection of mosquitoes with *Plasmodium* spp. in monkeys

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Equipment

enamelware or plastic pans
incubator (25 °C)

Materials and reagents

deionized water
baker's yeast
mosquito larval food
monkey/rat chow
lactalbumen (Sigma L-7252)
brewer's yeast (Sigma YBD)
10% sugar solution
cotton balls
blood source (rabbit/Guinea pig)
Parafilm

Preparation

Rearing anopheline mosquitoes

- Grind monkey or rat chow in a mill and sieve through a 60-mesh screen.
- Combine lactalbumen, inactive brewer's yeast, and chow (1:1:1).
- Store the mixture in a refrigerator.
- Allow mosquito eggs to hatch in a pan lined with strips of paper toweling.
- Feed a "pinch" of active baker's yeast on *Day 1*.
- On succeeding days, feed increasing amounts of mosquito larval food.
- Maintain the water depth at 2 to 3 cm.
- Every 3 or 4 days, split pans until *Day 8* to give approximately 1 larva/cm^2 of water surface.
- When pupae appear, strain and suspend larvae and pupae in ice water.
- Pour floating pupae into an emergence cup.
- Return the larvae to the rearing pan.
- Place the pupae in a cage for emergence.
- After they emerge, feed them a 10% sugar solution daily on a cotton ball.
- Beginning 3 days after emergence, offer them a blood meal from an anesthetized rabbit or guinea pig every 3 or 4 days. (Shave hair from the feeding area.).
- Three days after each blood meal, put a small bowl with water (1 to 2 cm depth) into the cage overnight to collect eggs.

Comments

Most commonly reared anopheline mosquitoes such as *Anopheles stephensi, A. atroparvus, A. quadrimaculatus, A. gambiae,* and *A. albimanus* will mate in cages; others such as *A. dirus* require force mating. The most suitable vector depends on the species and strain of *Plasmodium* being investigated.

Infection of mosquitoes by feeding on monkey

- Transfer female mosquitoes (3 to 7 days after emergence) to infection cages.
- Concentrate female mosquitoes by placing a warm hand to the side of the holding cage.
- Aspirate and transfer the mosquitoes to the infection cage.
- Partially starve mosquitoes overnight with 5% sugar solution.
- Anesthetize and immobilize the monkey on a restraining board using Velcro straps. (The center of the restraining board is cut out to allow the belly of the monkey to rest directly on top of the feeding cage.)
- Allow mosquitoes to feed to repletion (usually 10 to 15 min); remove and discard unfed mosquitoes.
- Store cartons of mosquitoes in a 25 °C incubator.
- Feed mosquitoes a 10% sugar solution during extrinsic incubation.
- Seven to 10 days after feeding (depending on the species of *Plasmodium*), dissect aliquots of mosquitoes to determine presence and numbers of oocysts.
- If oocysts are present, return undissected mosquitoes to the incubator.
- Dissect and examine salivary glands 10 to 18 days after feeding (depending on the temperature and species of *Plasmodium*).

Comments

Development of oocysts depends on the temperature and the species of *Plasmodium*. When incubated at 25 °C, the quotidian parasite *P. knowlesi* completes sporogonic development in 10 days; the tertian parasites *P. vivax, P. ovale, P. cynomolgi, P. gonderi, P. coatneyi, P. fragile, P. fieldi, P. simium*, and *P. simiovale* in 10 to 15 days; the quartan parasites *P. malariae, P. brasilianum*, and *P. inui* in 15 to 20 days.

Infection of mosquitoes by membrane feeding

- Transfer mosquitoes to infection cage (as above).
- Collect blood in heparin from the donor animal.
- Centrifuge blood at 1,500 rpm for 10 min.
- Remove plasma and replace it with serum or heparinized blood from an unexposed animal or human.
- Cover the feeding surface of a water-jacketed feeding bell with stretched Parafilm.
- Place the blood in the chamber.
- Commence circulation of water (37 °C).
- Lower the bell onto the netting of the feeding cage and allow mosquitoes to feed to repletion for 5 to 10 min.
- Remove and discard unfed mosquitoes.
- Feed mosquitoes a 10% sugar solution during extrinsic incubation.

Comments

Blood is collected in heparin, ACD, or is defibrinated with glass beads; EDTA inhibits infection. Transmission-blocking immunity often develops rapidly in monkeys. Removal of plasma and its replacement with serum or blood from an uninfected host allows continued mosquito infection if infectious gametocytes are present. Membrane feeding provides a means of assessing the transmission-blocking activity of immune sera by combining dilutions of sera from immunized

monkeys with infectious gametocytes. Reduction in oocyst densities measures transmission-blocking activity.

References

Earle WC, Perez M. 1932. Enumeration of parasites in the blood of malarial patients. J Lab Clin Med 17:1146-1168.

Rutledge LC, Ward RA, Gould DJ. 1964. Studies on feeding response to nutritive solutions in a new membrane feeder. Mosq News 24:407-409.

Sullivan JS, Strobert E, Yang C, Morris CL, Galland GG, Richardson BB, Bounngaseng A, Kendall J, McClure H, Collins WE. 2001. Adaptation of a strain of *Plasmodium vivax* from India to New World monkeys, chimpanzees, and anopheline mosquitoes. J Parasitol 87(6):1398-1403.

III. Experimental malaria: using bloodstage infections of rodent malaria

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Equipment

hemocytometer
light microscope
liquid N2 freezer

Materials and reagents

syringes and needles for i.p and i.v injections
microscope slides
cryovials (e.g. Nunc)
Plasmodium freezing medium: 15% Glycerol in RPMI with 5% fetal calf serum and heparin (10units/mL)
Giemsa stain (Gurr's improved Giemsa, BDH Laboratory Supplies)
phosphate buffer (Na_2HPO_4 - 1g/L; KH_2PO_4 – 0.7g/L)
acridine orange (AO) (optional)
heparinized tubes
methanol
frozen stabilate of rodent malaria (e.g. *Plasmodium chabaudi, P. berghei, P. yoelii* etc)

To make frozen stabilates

- Collect blood, from mice with ascending parasitemia, directly into parasite freezing medium at a ratio of 1mL blood to 4mLmedium.
- Working aseptically, mix the blood and aliquot (100 – 500 μL/tube) in cryotubes and freeze immediately in liquid nitrogen. (For short periods, the tubes can be frozen and stored at −70 °C.)

  NB. Parasite virulence can change after a number of *in vivo* passages, therefore it is important to start from a fresh stock of stabilates regularly.
  (Freeze down stocks of new stabilates regularly)

To start an infection

- Thaw a cryotube of parasites quickly at room temperature.
- Inject undiluted contents immediately i.p. into a naive recipient mouse. Dilute the stabilate 1:2 with PBS for i.v injections.
- Start monitoring parasites in peripheral blood after 3-4 days.

Counting parasitemia

- Make a thin blood film from tail blood, fix with methanol for 2 min, and stain with Giemsa (Dilute Giemsa stain1:10 in phosphate buffer).
- Count percent infected cells (100× oil immersion).
  (Instead of Giemsa staining, acridine orange (AO) staining of thin blood films is possible. After methanol fixing, add a small drop of AO on a slide, add a

coverslip, and count parasitemia under a light and UV-light microscope (see PARASITES, sections III:A, C).

Passage of infection into new mice

- Collect blood from the donor mouse during ascending parasitemia. Transfer 10 uL of tail blood into 5 mLchilled PBS and mix.
- Count parasitemia as above and the number of erythrocytes per microliter in a hemocytometer.
- Dilute the blood in sterile PBS to 5 x 10^5 infected erythrocytes per milliliter.
- Inject 200 μL i.p. or i.v. per mouse (10^5 infected erythrocytes per dose).
- Parasites normally appear in the blood stream after 2 to 3 days.

N.B.
If you inject i.v., only one-tenth of the i.p. dose is needed to obtain similar parasitemias.

Reference

Cox FEG. 1988. Major animal models in malaria research. *In:* Wernsdorfer WH, McGregor I, eds. Malaria: principles and practice of malariology. Vol. 2. Edinburgh: Churchill Livingstone; pp. 1503-1543

IMAGING

I. *In vivo* imaging of pre-erythrocytic forms of murine *Plasmodium* parasites

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Real-time *in vivo* studies of *Plasmodium* sporozoites within host tissues, will lead to a better understanding of how the parasite invades and then develops in host cells. In this method, we describe the observation of pre-erythrocytic parasites in the dermis and the liver of mice. At early time-points, parasites can be tracked from the time they arrive in the tissue until they invade cells. At later time-points, parasite development in host cells can be analysed.

Equipment

stereomicroscope equipped with epi-fluorescence
fluorescence microscope, equipped with objectives that have a long working distance (for higher penetration into the tissue), and coupled to a camera
microscope platform to hold animal

Materials and reagents

Plasmodium berghei and/or *P. yoelii* fluorescent sporozoites (GFP or RFP)
70% Ethanol
1x PBS
microscope glass slides
50 I.U. and 100 I.U. Insulin syringes (Terumo)
Eppendorf microtubes
1x PBS, 5% bovine serum albumin (BSA)
hemocytometer (CML)
SKH1 Hairless (dermis) or C57BL/6 (liver) mice, if possible Pentobarbital or a mix containing 2% Rompun (Xylazine, Bayer) and Imalgene 1000 (Ketamine, Merial)
24 x 60-mm cover slips
BSA coupled to a fluorochrome (Alexa 488, 555 or 647) (Invitrogen)
Specific to each tissue:
For skin: Double-sided Scotch-tape
Nanofil intradermic needles with 35G needles
3M Scotch-tape
For liver: Surgical equipment (tweezers, scissors, electro-cautery)
SuperGlue 3

Procedure

Selection of mosquitoes positive for salivary gland sporozoites (infection by natural bite)

- At least 24 hours before the bite session gently aspirate mosquitoes and then blow them into a tube on ice. Transfer the mosquitoes to a Petri dish on ice.

- Under a fluorescence stereomicroscope, determine which mosquitoes contain fluorescent parasites in the salivary glands and place the mosquitoes in a recipient covered with netting.
- Keep the mosquitoes at 21°C and 70% humidity without nourishment (sugar solution) until the bite session.

Dissection of mosquitoes to obtain salivary gland (SG) sporozoites

- Anaesthetise mosquitoes by placing on ice. Place 10 seconds in 70% ethanol then transfer to 1x PBS. Place 10-12 mosquitoes on a microscope slide.
- Under the stereomicroscope, determine which mosquitoes are positive for SG sporozoites.
- Place one needle on the thorax of the mosquito and one needle at the base of the head. Gently pull the head away from the body, removing the SG from the thorax.
- Cut the salivary duct freeing the SG from the head and place the SG in an Eppendorf microtube containing 20 μL of PBS 1x.
- Repeat this for around 50 mosquitoes.
- To determine the number of sporozoites, crush the SG, liberating the sporozoites. Take 1 μL of sporozoite suspension and add to 9 μl of 1x PBS, 5% BSA. Place the 10 μL in a hemocytometer and count the number of sporozoites
- It is preferable to also determine the percentage of sporozoites that are gliding (moving in circles) as this has been shown to be related with a good infectivity.

Anaesthesia of mice

- Inject i.p 100 μL of an appropriately diluted solution of Pentobarbital (50 mg/ 20 g mouse bodyweight). Anaesthesia with Pentobarbital reduces the chances of hyperventilation by the animal; however, the dose needed, is harder to control as the product is stocked in fat and then diffuses.
- A solution containing 2 volumes of Imalgene 1000, 1 volume of 2% Rompun and 5 volumes of 1x PBS can also be used but may lead to hyperventilation and the anaesthesia is generally considered to be of shorter duration. Inject i.p. 100 ul of this solution for a mouse weighing 20 g.

Infection of mice

- For the dermis, the infection is generally done in the ear, but another part of the body such as the abdomen can be used:

  By natural bite, once the animal has been anaethestised, hold it gently over the cage containing the positive mosquitoes until one comes to feed. Allow the mosquito to bite for 1 minute (if left longer, move the mouse regularly). Note the time the bite started and the location, for imaging afterwards. The mouse can be observed immediately in order to follow the sporozoites or at later time points for parasite development.

  By intradermal injection, after anaesthetising the animal, tape the ear (as flat as possible), internal face down, onto the double-sided tape and inject i.d., on the external face of the ear, 1 μL of the sporozoite suspension, in 3 to 5 different places. The animal is observed immediately or after different times after injection depending on what developmental stage of the parasite is to be observed.
- For the liver, the infection is different depending on whether an early time-point or a later time-point will be observed

For early time-points, once the animal has been placed on the microscope platform, the sporozoites are injected in the retro-orbital sinus, at the same time as the fluorescent BSA, and observed immediately.
For later time-points, the sporozoites are injected i.v. in the mouse tail vein. The mouse is observed from 45 hours after infection. The fluorescent-BSA is injected in the retro-orbital sinus just before imaging.

Exposure of the ear for imaging

- The animal is anaesthetised.
- A cover glass is Scotch-taped to the microscope platform.
- The mouse is placed on the platform so that the infected ear can be Scotch-taped to the cover glass. It is important that the ear is placed as flat as possible on the cover glass.

Exposure of the liver for imaging

- Anaesthetise the animal.
- Disinfect the abdomen with 70% ethanol. The animal is not pinned to a dissection board.
- Incise the skin 0.5 cm below the xiphoid process, and enlarge the opening to about 1 cm. Hairs that may have entered the incision must be removed with 1x PBS.
- Make a horizontal cut through the muscle layers. Do this carefully, with round-edged scissors, to avoid damaging the liver. This may lead to bleeding if capillaries are sectioned, the haemorrhage must be stopped immediately with the cautery and any blood removed, as this will perturb the observation.
- Gently push on the abdomen so that the lower tip of the left lobe appears. The incision must be big enough to allow the liver to extrude but small enough that it remains exposed and does not fall back into place. Place a drop of 1x PBS on the lobe so that it does not dehydrate during observation.
  Place several drops, about 5, of SuperGlue 3 on the skin around the exposed lobe and place the cover glass horizontally over the lobe. Push gently to stick the cover glass into position.
- Note: If it is difficult to extrude the liver lobe, but a sufficient amount is visible then the cover glass can be placed without emerging the lobe.

General procedures for imaging parasites in murine tissues

- Place the platform holding the animal on the microscope.
- Using the autofluorescence of the tissues (dermis of the ear or capsule of the liver), set an arbitrary z=0 at this focal point. For imaging the liver sinusoids, the retro-orbital injection should be done at this point.
- Then using the focus wheel, penetrate into the tissue until a zone with parasites is observed. Delimit the zone to be observed, and choose the z-step to be used (the smaller the z-step the higher the resolution but the slower the acquisition and the larger the files will be).
- The zone to be observed can be acquired in a single stack (per fluorochrome to be observed) over time. Depending on what will be observed, the space between stacks must be set up accordingly. For instance, sporozoites that are highly motile should be filmed continuously. In this case, this should be done for short periods (5-10 minutes) to avoid cell damage due to phototoxicity.

Analysis of acquired images

- Once the images have been acquired they can be analysed using the corresponding software or can be analysed using the open source software ImageJ. For the second option, it is best if images are in .tif format.
- Films can be made to visualise the parasite during the time-lapse; this allows the visualisation of sporozoite motility.
- 3D-reconstructions can also be processed to visualise the position of parasites in comparison with tissue structures. For instance, to determine whether a hepatic sporozoite localised in a liver sinusoid or in the hepatic parenchyma.

Different types of analysis possible

- Tracking of sporozoites within the different tissues.
- Localisation of sporozoites within different zones of the tissue.
- Analysis of interactions between sporozoites and host cells, for instance immune cells.
- Evaluation of merosome volume and release of merozoites.

References

Rogerio Amino, Sabine Thiberge, Béatrice Martin, Susanna Celli, Spencer Shorte, Friedrich Frischknecht, Robert Ménard. 2006. Quantitative imaging of *Plasmodium* transmission from mosquito to mammal. Nature Medicine 12:220-224.

Angelika Sturm, Rogerio Amino, Claudia van de Sand, Tommy Regen, Silke Retzlaff, Annika Rennenberg, Andreas Krueger, Jörg-Matthias Pollok, Robert Ménard, Volker T. Heussler. 2006. Manipulation of host hepatocytes by the malaria parasite for delivery into liver sinusoids. Science 313:1287-1290.

Rogerio Amino, Sabine Thiberge, Samantha Blazquez, Patricia Baldacci, Olivier Renaud, Spencer Shorte, Robert Ménard. 2007. Imaging malaria sporozoites in the dermis of the mammalian host. Nature Protocols 2:1705-12.

Sabine Thiberge, Samantha Blazquez, Patricia Baldacci, Olivier Renaud, Spencer Shorte, Robert Ménard, Rogerio Amino. 2007. In vivo imaging of malaria parasites in the murine liver. Nature Protocols 2:1811-08.

II. Imaging of the blood stage *Plasmodium falciparum* merozoite during erythrocyte invasion

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This method outlines a procedure for immunofluorescence assay (IFA) of fixed samples of *Plasmodium falciparum* merozoites invading human erythrocytes, allowing high-definition exploration of parasite-erythrocyte interactions on multiple microscopy platforms.

Equipment

- Standard *P. falciparum* culture facilities including laminar flow hood and centrifuges (large and benchtop micro-centrifuge)
- A magnet for cell separation, such as VarioMACS^{TM} magnet system (Miltenyi Biotec) and columns (we use CS type).
- A basic orbital shaker such as the IKA MS 3 (IKA® Laboratory)

Materials and Reagents

Where unspecified, reagents should be available from commonly used laboratory chemical or equipment distributers (e.g. Sigma-Aldrich).

- Trans-epoxysuccinyl-L-leucylamido-(4-guanidino)butane (E64): 10 mM (1000x) stock made up in deionized water and frozen at -80°C.
- Complete culture medium: RPMI-Hepes (RPMI-1640, 25 mM Hepes, 200μM Hypoxanthine, 20 μg/mL Gentamicin) supplemented with 5.8 mL 3.6% $NaHCO_3$ and 10 mL 5% albumax (dissolved in RPMI-Hepes) per 100 mL.
- Incomplete culture medium: RPMI-Hepes (RPMI-1640, 25 mM Hepes, 200μM Hypoxanthine, 20 μg/mL Gentamicin) supplemented with 5.8 mL 3.6% $NaHCO_3$ per 100 mL.
- 10 mL disposable plastic syringe.
- 1.2 μm syringe filter (Sartorius, 17593).
- Freshly drawn human erythrocytes, washed in incomplete culture medium and resuspended at 50% haematocrit in the same medium.
- Fixative solution: For 2x stock, 8% paraformaldehyde, and 0.015% glutaraldehyde in HT-PBS.
- Permeabilisation solution: 0.1% Triton X-100 in HT-PBS.
- Blocking solution: 3% Bovine Serum Albumin (BSA) in HT-PBS, 0.2 μm filtered.
- Primary and secondary (fluorophore conjugated) antibodies: diluted appropriately in blocking solution.
- Coverslips – high performance type #1.5 (e.g. Carl Zeiss).
- Glass slides
- 1% polyethyleneimine (PEI) solution
- VectaShield (Vector Laboratories) + 0.2 ng/ μL 4',6-diamidino-2-phenylindole (DAPI).
- Quick drying nail varnish or VALAP (1:1:1 mixture of Vaseline, lanolin and paraffin that requires pre-heating to melting before being brushed lightly around coverslips to seal).

Methods

Magnet Purification of schizont stage parasites

- Purify schizonts from at least 90mL of >3% parasitemia, 4% haematocrit, highly synchronous late stage parasite culture by passing through a magnet column (set up as described by manufacturer) at a slow but steady dripping rate. Note, synchronicity is one of the keys to success of this method and all parasites must be within 6-8 hours of rupture (or ring stages) at the point of purification.
- Wash thoroughly with ~6 column volumes (30 mL) of warm medium.
- Remove column and elute in 4-6 column volumes (20-30 mL) of warm culture medium.

Isolation of merozoite invasion

- Add E64 to 10 μM final concentration. Note, if E64 is added to parasites <~40 hrs post invasion, this kills the parasites and they can block the syringe filter.
- Return to culture for 6-8 hrs.
- At a time when >50% parasites (higher is better) appear to be mature PVM-enclosed merozoite structures (PEMS) pellet at 1900 g for 5 min.
- Resuspend pellet in a minimal volume of ~750 μL warm incomplete culture medium (higher if sample appears very dark black to prevent clogging during filtration). Carry over of a small amount of complete culture media is fine.
- Push the resuspended parasites through a 1.2 μm filter. Some loss in the filter is inevitable. The resulting filtered merozoites in solution will survive best at RT and have a half-life of around 5-10 min.
- Add 500 μL of this mix to a 1.5 mL microtube containing 50 μL (or less for higher percentage of invasion) of 50% haematocrit washed blood and briefly mix.
- Place immediately in shaker at ~1000 rpm at 37˚C.
- After desired period (1 min 45 sec for invading parasites, longer for post-invasion), remove from shaker, add 2x fixative solution (550 μL) to the tube (s), mix by inverting and place on a roller at room temperature (RT) for 30 min.
- After 30 min spin at 2000 rpm in bench top microfuge for 3 min
- Remove supernatant and replace with 1 mL of permeabilisation solution and place on roller for 10min.
- Spin again, and resuspend in 1 mL blocking solution. Block minimally 1 hr at RT or O/N at 4˚C. This sample can be kept at 4˚C for up to one month.

Preparation of invading merozoites for imaging

- Spin down ~50-100 μL of blocked sample at 2000 rpm in bench top microfuge for 1 min.
- Remove supernatant and resuspend in minimally 100 μL blocking solution with appropriately diluted primary antibodies. Note that invasion preparations invariably contain a mixture of attached, invading and just invaded merozoites. As such imaging of a marker antigen (such as a tight junction component) is essential to determine quality of preparation and timing of events captured.
- Incubate on a roller at RT for 1 hr.
- Spin at 2000 rpm, remove supernatant and wash x3 in 500 μL HT-PBS, 10 min at RT on roller.
- Remove supernatant and resuspend in minimally 100 μL blocking solution with appropriately diluted secondary antibodies conjugated to fluorophores.

- Incubate on a roller at RT for 1hr. Do not expose to light from this point onwards.
- Spin at 2000 rpm, remove supernatant and wash x3 in 500 µL HT-PBS, 10 min at RT on roller.
- Meanwhile, very briefly flame an appropriate number of coverslips and coat with 1% PEI solution.
- Resuspend the remaining sample pellet in 300-500 µL (highly diluted samples provide lower density samples for ease of imaging) and pipette 100 µL on to the dried coverslip.
- Settle for 30 min at RT.
- Holding the cover glass with forceps, wash in HT-PBS, dry by placing the corner of the glass on a lab wipe, then invert onto a glass slide with 15 µL of VectaShield + 0.2 ng/µL DAPI on it.
- Press firmly on the cover glass to squeeze any excess mounting media out and wipe excess away with a lab wipe.
- Seal using commercial quick drying nail varnish or VALAP.
- Image

References

This method is derived from:
Boyle, M. J., Wilson, D. W., Richards, J. S., Riglar, D. T., Tetteh, K. K., Conway, D. J., Ralph, S. A., Baum, J., and Beeson, J. G. (2010) Isolation of viable *Plasmodium falciparum* merozoites to define erythrocyte invasion events and advance vaccine and drug development. Proc Natl Acad Sci USA 107, 14378-83. [merozoite isolation]

Riglar, D. T., Richard, D., Wilson, D. W., Boyle, M. J., Dekiwadia, C., Turnbull, L., Angrisano, F., Marapana, D. S., Rogers, K. L., Whitchurch, C. B., Beeson, J. G., Cowman, A. F., Ralph, S. A., and Baum, J. (2011) Super-Resolution Dissection of Coordinated Events during Malaria Parasite Invasion of the Human Erythrocyte. Cell Host Microbe 9, 9-20. [merozoite high-definition imaging]

Tonkin, C. J., van Dooren, G. G., Spurck, T. P., Struck, N. S., Good, R. T., Handman, E., Cowman, A. F., and McFadden, G. I. (2004) Localization of organellar proteins in *Plasmodium falciparum* using a novel set of transfection vectors and a new immunofluorescence fixation method. Mol Biochem Parasitol 137, 13-21. [general immunofluorescence protocol]

III. Fixation protocols for transmission electron microscopy

III:A. Fixation of tissue samples

Method developed by ***Dr. Masamichi Aikawa***

Please note: Use only glutaraldehyde of high purity, "EM-grade". Purchase only small amounts at any one time, store them at –20 °C, and use them as soon as possible. Also, use only paraformaldehyde powder. It is important to dissolve the paraformaldehyde in alkaline buffer before adjusting the cacodylate buffer to neutral pH.

Please note: **ALL CHEMICALS used in this protocol ARE EXTREMELY TOXIC.**

Solutions

For the solutions below:

- Dissolve paraformaldehyde in double-distilled water in a FUME HOOD.
- Stir and heat the solution until it reaches 60 °C.
- Add 1.0 N NaOH dropwise (1 to 2 drops) until the milky white solution turns clear.
- Cool this solution before adding cacodylate and glutaraldehyde.
- Adjust the pH with 1.0 M NaOH or 2 M HCl.
- Store solutions at 4 °C in dark bottles and use within 1 month or before a white precipitate appears.

Fixation buffer, 100 mL:

0.1 M sodium cacodylate (dimethylarsinic acid sodium salt trihydrate for synthesis), 1% glutaraldehyde, 1% paraformaldehyde (pH 7.4):

1 g paraformaldehyde (> 95% pure)
2.2 g sodium cacodylate
1.4 mL70% glutaraldehyde (1.96 mL if 50% GA stock), EM-grade
For adjusting the pH to 7.4, use 2 M HCl. Adjust volume to 100 mL using double-distilled water

Fixation storage buffer, 100 mL:

0.1 M sodium cacodylate, 0.1% glutaraldehyde, 0.1% paraformaldehyde (pH 7.4):

0.1 g paraformaldehyde (> 95% pure)
2.2 g sodium cacodylate
0.14 mL 70% glutaraldehyde (0.2 mL if 50% GA stock), EM-grade
For adjusting the pH to 7.4, use 2 M HCl. Adjust volume to 100 mL using double-distilled water.

Procedure

- Cut pieces of tissue to a maximum size of 0.5 cm^3.
- Immediately immerse the tissue cube in fixation buffer and leave it for 30 min.
- Aspirate the fixation buffer and fill the tube with fixation storage buffer. Fill the tube to the top to avoid contact with oxygen which will cause formalin crystals in the sample.
- Close the tube carefully and seal it with Parafilm.
- Store the samples at 4 °C. When being transported, the samples do not need to be at 4 °C if that is not possible.

Reference

Scholander C, Treutiger CJ, Hultenby K, Wahlgren M. 1996. Novel fibrillar structure confers adhesive property to malaria-infected erythrocytes. Nat Med. 2(2):204-208.

XI:B. Fixation of erythrocytes for immuno-electron microscopy

*Method developed by **Dr. Masamichi Aikawa***

Equipment

centrifuge

Materials and reagents

parasite culture with desired parasitemia
RPMI 1640
0.1 M phosphate buffer (pH 7.4)

Fixation solution, 100 mL (1% paraformaldehyde, 0.2% glutaraldehyde, 0.1 M phosphate buffer (pH 7.4):

Prepare phosphate buffer stocks (Na_2HPO_4 and NaH_2PO_4), then mix for 0.2 M (pH 7.4) according to Maniatis or "Current Protocols in Molecular Biology".

Mix 1.0 g paraformaldehyde in 50 mL of double-distilled water. In a FUME HOOD, stir and heat the solution until it reaches 60 °C, add 1.0 N NaOH dropwise (1 to 2 drops) until the milky white solution turns clear.

50 mL 0.2 M phosphate buffer

0.4 mL glutaraldedyde (50% stock)

This fixing solution lasts for 2 months when stored at 4 °C.

Procedure

- Grow a parasite culture until desired parasitemia is reached.
- Cool RPMI 1640, 0.1 M phosphate buffer, and fixing solution to 4 °C.
- Wash cells in RPMI 1640 twice and once in cold 0.1 M phosphate buffer (pH 7.4).
- Fix samples in cold fixation solution for 10 min at 4 °C.
- Wash the sample three times in cold 0.1 M phosphate buffer. If using microfuge tubes, spins at 2,500 rpm for 1 min at 4 °C suffice.
- If shipping samples, fill the tubes completely with 0.1 M phosphate buffer, cap them well, and ship them on ice by express.
- If shipping antibodies, add sodium azide for a final concentration of 0.01% and send them on ice by express.

References

Method developed by Dr. Masamichi Aikawa.

Ausubel FM, et al., eds. Current protocols in molecular biology. New York: Wiley-Interscience; 1988-1999.

Quakyi IA, Matsumoto Y, Carter R, Udomsangpetch R, Sjölander A, Berzins K, Perlmann P, Aikawa M, Miller LH. 1989. Movement of a falciparum malaria protein through the erythrocyte cytoplasm to the erythrocyte membrane is associated with lysis of the erythrocyte and release of gametes. Infect Immun 57(3):833-839.

Sambrook J, Fritsch EF, Maniatis T. 1989. Molecular cloning: a laboratory manual. 2nd ed. Cold Spring Harbor, New York: Cold Spring Harbor Laboratory Press.

IV. Preparation for Negative Staining

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Material

EM grid covered with Formvar (you can ordered from several companies, e.g. Plano and EMS) or make yourself the plastic covering
Ultra fine tweezers
Fixative solution – 2% glutaraldehyde
Filter paper (Whatman® number 1)
Staining solution – 1% Uranyl acetate in water

Obs: This solution is better prepared freshly and protected from light (the solution is light sensitive).

Procedure

Fix the sample (it can be sporozoites or merozoites, for example) for 20 minutes
Wash the sample with PBS to remove the fixative
Place 5-10 µL of the sample onto the EM grid that is being held by tweezers (as shown in Figure 1A) and incubate for 10 minutes
With the filter paper, blot the liquid excess
Add the uranyl acetate solution (about 10-15 µL) and at the same time blot the liquid (as shown in Figure 1B)
Store the grids in a gridbox and go to a transmission electron microscope for observation

Note – Glutaraldehyde and uranyl acetate are toxic solutions. Do use gloves while manipulating.

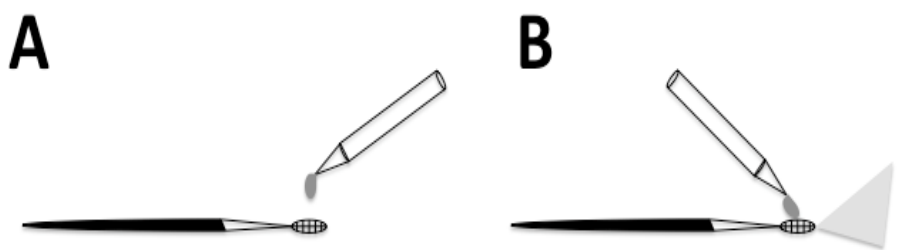

Figure 1: A - With a pipette, place the sample onto the grid, and afterwards blot the liquid excess. B – While applying the staining solution, blot the liquid with a filter paper.

Negative staining is a suitable technique for fast visualization of samples for transmission electron microscopy. However, if the interest is intracellular architecture, classical transmission electron microscopy is required. Negative staining can also be used to observe the cytoskeleton arrangement (Aikawa M.,1967). For this purpose, extract the membranes of your sample (e.g. 1% Triton X-100 or NP-40 detergent

solution for 15 minutes prior to fixation) (Lemgruber et al. 2010). Also, immune-cytochemistry detection can be done in negative staining. After fixation, proceed as a normal with an immunocytochemistry protocol. After secondary antibody incubation, wash the sample; place the sample onto a Formvar-coated grid and proceed as normal for negative staining (Lemgruber et al., 2010). During observation in the microscope, several pitfalls can be encounter, e.g. the plastic film is disrupted or disrupting during observation.

References

Aikawa M. (1967). Ultrastructure of the pellicular complex of *Plasmodium fallax*. J Cell Biol 35:103-113.

Lemgruber L, Kloetzel JA, Souza W, Vommaro RC. (2010). *Toxoplasma gondii*: further studies on the subpellicular network. Mem Inst Oswaldo Cruz 104:706-709.

V. Preparation for Cryo-electron tomography

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The procedures for preparation and imaging and tomography of whole cells preserved in hydrated state require additional precautions over that of standard cryo-samples of isolated particles (Dubochet et al., 1988). In this manual we highlight these precautions and special treatments.

Cryo-preparation:

Material

EM holey-carbon grid (commercial: Quantifoil, Lacey that you can ordered from several companies, e.g. Plano and EMS; or home made ones)
Ultra fine tweezers
Colloidal gold, normally of 10 nm (you can purchase from several companies, e.g. Sigma)
Filter paper (with relatively rough fibers – we prefer Whatman® number 1)
Liquid Nitrogen
Ethane

Equipment

A homemade or a commercial plunging device for rapid freezing. We use one produced by the workshop at EMBL in Heidelberg (price: approx. 800 €)

Procedure

- Make sure that the carbon surface of a holey carbon coated EM grid is not strongly hydrophobic (you can achieve that by e.g. discharging the grids in a glow discharge device or by soaking the grids in acetone for several hours and subsequent drying in air)
- Pipette an aliquot (3-8 µL) of a sample (sporozoites, merozoites, or infected-red blood cells) on the carbon side of a holey EM grid that is being held with tweezers as shown in Figure 1 (use either self closing tweezers or close them with a rubber or silicon ring)
- Add colloidal gold (approx. 1 µL) from the back side of the grid (distribute the liquid across the grid, make sure that both sides are completely wetted and the contents mixes up)
- Wait several minutes for sample adhesion to the grid surface, and for relaxation of the cells after pipetting

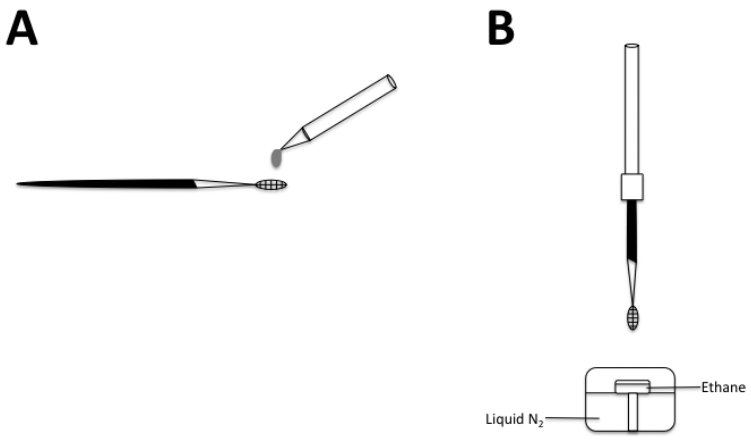
Notes: If necessary you can increase the sample concentration directly on the grid by slowly pipetting the sample several times up and down from the backside of the grid.

You can prepare this way several grids hold in tweezers – to be frozen in one series

- Mount the tweezers in the plunging device
- Blot off the excess of liquid with filter paper from the back side of the grid (gently press the filter paper against the grid at the side you put gold on, not where you applied the cells) for 1-3 seconds (blotting time depends on several factors, like sample viscosity and ambient humidity), wait another second for evaporation of excess of water that facilitates formation of very thin film of liquid around the cells, release the plunging device and freeze the sample into liquid ethane cooled down with liquid nitrogen to the temperature near its melting point (-183°C). The gravity-driven plunging device should be used to assure the 'entrance velocity' of the grid into cryogenic liquid of at least 1m/s

Obs

- Liquid ethane solidifies quickly (melting point at -183°C), when cooled down with liquid nitrogen (boiling point at -196°C). The grids might smash against solid ethane. Thus when preparing several grids in a row, one has to keep ethane at liquid state by gentle blowing small amounts of ethane gas into the ethane recipient.
- You can store frozen samples in a liquid N_2 container (free of condensing water) for any length of time before scoping them in a cryo-electron microscope.



A video showing the procedure of rapid freezing can be seen in Chen et al. (2010).

Imaging frozen, fully hydrated samples:

The grids are mounted in a cryo-holder, inserted into a microscope and kept at cryogenic temperatures by liquid nitrogen or liquid helium. Cryo-samples can never be warmed up above so called 'de-vitrification temperature', by which originally vitreous water would crystallize into cubic and hexagonal ice and destroy the fine structure. In addition, a particular precaution has to be taken to prevent an exposure of a grid to ambient humidity, which will cause an immediate contamination with condensing ice-crystals. The manufacturers of cryo-instrumentation provide with detailed manuals describing the correct handling of cryo samples.

When viewing and imaging cryo-samples in electron microscope the following rules should be followed: Fully hydrated sample is particularly susceptible to damage by corrosive electron beam. Thus the minimum of electron dose should be applied when

viewing and imaging the cryo grids (Dubochet et al., 1988). As an effect of rather limited control by blotting of liquid during cryo-specimen preparation one can often see the layers of water too thick for electrons to transmit through and to form the image. The opposite is not ideal either; in the strongly over-blotted areas of grids the cells are either squeezed between closely apposing water surfaces or even dried out before freezing. The optimal areas for imaging should have uniform thickness of vitrified water. The ice thickness should match that of the thickness of specimen.

You can record a single image of the specimen, the focal series or the tilt series – notice that there is a limited electron dose you can apply on a specimen before destroying it by electron beam. There is roughly linear correlation between the electron dose on frozen-hydrated specimen and the resolution in resulting images. In practical terms: when aiming on 1 nm resolution one should not irradiate the specimen with doses exceeding 1,000 electrons per 1 nm^2 of specimen (or 10 electrons / $Å^2$). By 5-6 nm resolution the electron dose could be of the range of 8,000-10,000 electrons / nm^2 (or 80-100 electrons / $Å^2$). For the calibrations and measurements of electron doses consult the manuals provided by the manufacturers of microscopes.

Cryo-electron tomography:

Tomographic tilt series consist typically of 60-80 images recorded in the intervals of 2° (1.5°-3°) and cover a range of 120°-140° (between +- 60º and +-70º). Notice that the maximum dose of electrons has to be distributed evenly throughout the series, resulting in strongly underexposed images with low signal to noise ratio. Nevertheless, the signal is considerably improved by further merging the tilted images in 3D reconstruction (the sampling theorem – Lucic et al. 2005). The actual reconstruction is performed in two stages: 1. Alignment of images using either gold particle as fiducial marker or by cross-correlation; and 2. Back-projecting the aligned images in a three-dimensional reconstruction. Software for tomographic reconstructions and volume processing are available online (e.g. IMOD, EM, TOM). More on this subject can be found in books dedicated for electron tomography (Frank, 2005; McIntosh, 2007). Applying specific filtering and de-noising procedures could enhance the visual factor of tomograms. Further processing of tomograms, data mining and visualization could be done on specially developed software packages, such as TOM, SPIDER, IMOD, and AMIRA®.

References:

Chen S, McDowall A, Dobro MJ, Briegel A, Ladinsky M, Shi J, Tocheva EI, Beeby M, Pilhofer M, Ding HJ, Li Z, Gan L, Morris DM, Jensen GJ. (2010). Electron cryotomography of bacterial cells. J Vis Exp 39 pii: 1943.

Cyrklaff M, Kudryashev M, Leis A, Leonard K, Baumeister W, Menard R, Meissner M, Frischknecht F. (2007). Cryoelectron tomography reveals periodic material at the inner side of subpellicular microtubules in apicomplexan parasites. J Exp Med 204:1281-1287.

Cyrklaff M, Sanchez CP, Kilian N, Bisseye C, Simpore J, Frischknecht F, Lanzer M. (2011). Hemoglobins S and C interfere with actin remodeling in *Plasmodium falciparum*-infected erythrocytes. Science 334:1283-1286.

Dubochet J, Adrian M, Chang JJ, Homo JC, Lepault J, McDowall AW, Schultz P. (1988). Cryo-electron microscopy of vitrified specimens. Q Rev Biophys 21:129-228.

Frank J, ed (2005). Electron Tomography, 2nd edition. (Springer, New York).

Henrich P, Kilian N, Lanzer M, Cyrklaff M. (2009). 3-D analysis of the *Plasmodium falciparum* Maurer's clefts using different electron tomographic approaches. Biotechnol J 4:888-894.
Kudryashev M, Lepper S, Stanway R, Bohn S, Baumeister W, Cyrklaff M, Frischknecht F. (2010). Positioning of large organelles by a membrane- associated cytoskeleton in *Plasmodium* sporozoites. Cell Microbiol 12:362-371.
Lucić V, Förster F, Baumeister W. (2005). Structural studies by electron tomography: from cells to molecules. Ann Rev Biochem 74: 833-865.
McIntosh JR, ed (2007). Methods in Cell Biology, vol 79 - Cellular Electron Microscopy (AmericanPress, San Diego).
Lemgruber L, Kudryashev M, Dekiwadia C, Riglar DT, Baum J, Stahlberg H, Ralph SA, Frischknecht F.(2013). Cryo-electron tomography reveals four-membrane architecture of the Plasmodium apicoplast. Malar J 12:25.

VI. Calcium signaling in malaria parasites

SINGLE CELL IMAGING AND FLUORIMETER ASSESMENT OF CALCIUM IN SUBCELLULAR COMPARTMENTS OF MALARIA PARASITES

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P. falciparum and other plasmodia species, such as *P. berghei*, *P. chabaudi* and *P. yoelii* utilize Ca^{2+} as an intracellular messenger. Here we describe our methods for single cell imaging to monitor changes in cytosolic calcium in these *Plasmodium* species.

Equipment:
Confocal microscope equipped with Argon laser (488nm and 543nm wavelength emission).
Imaging chamber and mounting unit
Centrifuge
Nitrogen charged UV laser
Spectrofluorimeter with cuvette adaptor (RF5301-PC, Shimadzu)

Materials and Reagents
Rhod-2 AM (Invitrogen)
Powdered cellulose, medium pore (Whatman CF11)
10 mL syringe (BD)
Glass wool (Merck)
50 mM Glass Bottom Dishes (Mattek Corporation, P50G-1.5-14-F)
Quartz cuvette, 1.5 mL (Hellma) with fitting magnetic stirrer
Fluo-3-AM (Invitrogen)
Fluo4-AM (Invitrogen)
Poly-L-Lysine
Borosilicate glass coverslips (to fit imaging chamber)
ci-IP_3 cell permeant (siChem)*
Pluronic acid
Mag-Fura-2 (Invitrogen)
FRET peptides for proteolysis experiments
MOPS Buffer: 116 mM NaCl, 5.4 mM KCl, 0.8 mM $MgSO_4$, 5.5 mM D-glucose and 50 mM MOPS, 2 mM $CaCl_2$, pH 7.2.
HBSS: 25 mM HEPES, 121 mM NaCl, 5 mM $NaHCO_3$, 4.7 mM KCl, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4$, 2.0 mM $CaCl_2$, 10 mM glucose, 0.04 mM probenecid, and 0.25% (w/v) fatty acid-free BSA, pH 7.4
PBS: 137 mM NaCl, 2.7 mM KCl, 4.3 mM Na_2HPO_4, 1.4 mM NaH_2PO_4
ACD: 60 mM Trisodium citrate dehydrate, 30 mM citric acid, 166 mM D-glucose
* Equipment and reagents required only for photorelease of IP_3 protocol (see below).

Protocols:

I *Calcium imaging in isolated parasites*

- Collect *P.falciparum* parasitized RBCs (parasitemia 3-8%) by centrifugation (1,500 g; 5 min; RT), resuspend in phosphate buffered saline (PBS) and wash three time in PBS.
- Isolate parasite by lysing RBCs (10^8 cells) with 10 mg/ml^{-1} saponin in PBS.
- Pellet cells (2,000 g) to remove red cell membrane material and wash twice in PBS by centrifugation (2,000 *g* at RT).
- Resuspend parasites in MOPS buffer (wash twice) and resuspend in MOPS containing probenecid (40 μM)[1].
- Load parasites in suspension with Fluo-4 AM (to a final concentration of 5 μM) and incubate for 50 min at 37°C.
- After loading pellet cells (2,000 g) and wash with MOPS to remove excess dye.
- Pipette cells onto borosilicate glass coverslips previously coated with poly-L-lysine (50 μg/ml). Incubate at RT for 15 mins then wash (gently) to remove cell which did not adhere.
- Transfer coverslip to imaging chamber and add MOPS (in the presence of probenecid).
- Mount imaging chamber onto stage of confocal microscope coupled to a CCD camera.
- Detect Fluo4-AM fluorescence by excitation at 488 nM (Argon laser) and emission collection over 510 nM. We perform imaging experiments at room temperature at a frame rate of 0.3-1 htz.
- Apply drug or test compound to the bath; increases in Fluo4-AM fluorescence reflect elevations in cytosolic Ca^{2+}.

II *Calcium imaging in parasitized RBCs*

- *Culture P. falciparum to a parasitemia of 5-10%.*
- *Wash P. falciparum* infected erythrocytes three times with HEPES-buffered saline solution (HBSS) by centrifugation (1,500 g, 5 min, RT).
- Enrich parasitized population by aspirating loosely pelleted cells from the top.
- Resuspend parasitized RBCs in HBSS containing probenicid (40 μM) and load with Fluo4-AM for (5 μM, 1 hr, 37ºC).
- Pellet cells (1,500 g, 5 min, RT) and wash twice with HBSS to remove excess dye. Resuspend in HBSS containing probenicid.
- Pipette cells onto borosilicate glass coverslips previously coated with poly-L-lysine (50 μg/ml). Incubate at RT for 15 mins then wash (gently) to remove cells which did not adhere.
- Transfer coverslip to imaging chamber and add HBSS (in the presence of probenecid).
- Mount imaging chamber onto stage of confocal microscope coupled to a CCD camera.
- Detect Fluo4-AM fluorescence by excitation at 488 nM (Argon Laser) and emission collection over 510 nM. We perform imaging experiments at room temperature at a frame rate of 0.3-1 htz.
- Apply drug or test compound to the bath and monitor changes in Fluo4-AM fluorescence.

III *Photorelease of caged IP_3 parasitized RBCs*

- Culture and collect parasitized RBCs as described above.
- Co-load cells in suspension with caged-IP_3 (2 µm; siChem (stock solution made up with 10% pluronic acid DMSO)[3] and Fluo4-AM (5 µm; 37 °C) for 1hr. (in the presence of probenecid).
- Plate cells on coverslips as above and mount on stage of a confocal microscope coupled to a UV laser.
- To achieve the requisite temporal resolution for rapid Ca^{2+} changes elicited by photorelease of caged IP_3 we image using an Axiovert2000 (Zeiss) spinning disc confocal microscope and acquire data between 2-5 Hz.
- Uncage IP_3 using a UV laser. We employ a nitrogen charged UV flash lamp (Photon Technology International) guided through the objective (NB this configuration requires a UV corrected objective).
- Detected IP_3-dependent elevations is *P. falciparum* cytosol Ca^{2+} as above, by excitation of Fluo4-AM with at 488 nM and collection of emission > 510 nM.

IV *Preparation of murine parasites for calcum dynamics experiments*

- We maintain *Plasmodium chabaudi, Plasmodium berghei and Plasmodium yoelii* in Balb/c mice by transfer infection.
- The cellulose column is prepared by adding 5 mL of PBS to 0.6g of cellulose powder. The mixture is poured into a 10 mL syringe with glass wool on the bottom and excess PBS is drop out. The flow is stopped when PBS is about to reach the cellulose column with the aid of a plastic hose and a clip.
- Infected RBCs (around 30% parasitemia) are taken from malaria parasites in a syringe containing 1 mL ACD.
- RBCs are washed three times (300 g, 5 minutes).
- When necessary for the experiment, parasites are isolated from RBCs using 10 µg/mL saponin, in 50 mL PBS, at RT.
- Parasites are collected by centrifugation at 12800g, 10 minutes, at 4 °C.
- Parasites are washed twice in MOPS buffer (spin at 12800g) and resuspended in the same buffer.

V *Calcium imaging of parasite mitochondria* in murine parasites

- Rhod-2 AM is diluted in DMSO at the concentration of 5 mM and aliquoted into 5 µL stock solutions.
- Isolated parasites are resuspended in 1 mL MOPS buffer containing 5 µM Rhod-2 AM and 2 mM probenecid[4].
- Parasites are incubated for 50 minutes at 37 °C in water bath.
- Parasites are washed two times in MOPS buffer containing 40 µM probenecid and resuspended in the same buffer.
- Parasites are plated onto Mattek 50 mm glass bottom dishes pre coated with poly –L-lysine solution.
- Parasites are observed under the confocal microscope using 488 nm and 543 nm lasers and collecting dluorescence with longpass filter 560 nm and band pass filter of 505 nm – 530 nm.
- Typically, images are taken each second (1 Hz frequency).

VI *Calcium dynamics using a spectrofluorimeter*

- Isolated parasites are loaded with Fluo-4 as described above.
- Parasites are used are used at 10^7-10^8 parasites per 1.5 mL cuvette, containing 1 mL MOPS buffer with 2mM of probenecid.
- The mixture is kept under agitation with the magnetic stirrer, at 37 °C.
- Fluo-4 is excited at 505 nm and emmited fluorescence is collected at 530 nm.
- Fluorescence is obtained tipically with the frequency of 1 Hz.

VII *Simultaneous calcium measurement in the cytosol and parasitophorous vacuole of P. falciparum parasites.*

- *Plasmodium falciparum* parasites, infecting red blood cells, synchronized at the schizont stage are loaded with Mag-Fura-2 at 10 µM, in MOPS buffer (1mL), at 37 °C.
- Invasion is monitored in cultures via Giemsa-stained smears.
- Upon invasion, parasites are held at 4 °C to prevent endocytosis of Mag-Fura-2 by the parasite. Mag-Fura-2 should stain the parasitophorous vacuole.
- Parasites at the early ring stage are loaded with Fluo-3 AM at 10µM, in MOPS buffer (1mL) in the presence of 2 mM probenecid, for 1h.
- Parasites are washed with MOPS buffer and plated onto glass bottom dishes, previously treated with poly-lysine (200 µL volume).
- Parasites are observed under a confocal microscope. The ratiometric dye Mag-Fura-2 is excited at 351 nm and 375 nm and emitted fluorescence is collected between 475-525 nm. Fluo-3 is excited at 488 nm and emitted fluorescence is collected with a bandpass filter between 505-530 nm[5].
- Images are taken with the frequency of 1 Hz.

VIII *Simultaneous assessment of proteolysis and calcium in the parasite cytosol*

- Murine parasites are isolated and loaded with Fluo-4 as described above.
- Parasites are plated onto poly-lysine treated glass coverslips in 200 µL MOPS buffer.
- FRET peptides containing the fluorescent group ortho-aminobenzoic acid (Abz) and the quencher group EDDnp (ethylene diamine-2-4-dinitrophenyl) at 10 µM are added to the isolated parasites, already plated onto poly-lysine treated glass coverslips just prior to observation under the microscope or fluorescence assessment by spectrofluorimeter.
- The FRET peptide is excited at 351 nm and emitted fluorescence is collected with band-pass filter between 387-470 nm. Fluo-4 is excited at 488 nm and emitted fluorescence collected between 505-530 nm.
- Images are taken with the frequency of 1 Hz.

Notes

- Inhibition of organic anion transporters prevents Fluo4-AM from being pumped from the *P. falciparum* cytosol into the parasitophorous vacuole (PV). The high Ca^{2+} concentration in the PV result in a saturated Fluo4-AM signal and precludes detection of cytosolic Ca^{2+} responses.
- To enhance cell viability cells can be incubated in screw cap tubes filled with (95% Nitrogen; 3% oxygen; 2% Carbon dioxide) for the dye/caged compound loading period.

- To enhance Ca^{2+} indicator loading we premix an aliquot of stock Fluo4-AM (1mM in DMSO) with 10% pluronic acid to disperse the dye before addition of loading buffer. Add 10% pluronic acid to a final concentration of 0.02%.
- Simultaneous loading with Rhod-2 AM and Fluo-4 AM can be performed, by incubating the parasite in MOPS buffer containing 2mM probenecid for 50min at 37 °C in a water bath.
- Alternatively, Mag-Fura-2 fluorescence can be assessed with the use of a spectrofluorimeter, using the excitation wavelengths of 345/380 and collecting the emitted fluorescence at 510 nm.

References

Beraldo FH, Almeida FM, da Silva AM, Garcia CR. 2005. *J Cell Biol* 170: 551-7
Gazarini ML, Garcia CR. 2004. *Biochem Biophys Res Commun* 321: 138-44
Gazarini ML, Thomas AP, Pozzan T, Garcia CR. 2003. *J Cell Biol* 161: 103-10
Alves E, Bartlett PJ, Garcia CR, Thomas AP. *J Biol Chem* 286: 5905-12
Farias SL, Gazarini ML, Melo RL, Hirata IY, Juliano MA, et al. 2005.*Mol Biochem Parasitol* 141:71-9

IMMUNOCHEMISTRY

I. Studies of the *Plasmodium falciparum*-infected erythrocyte surface

I:A. Surface iodination of PRBC (Lactoperoxidase method)

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Materials and reagents

parasite culture at 5% hematocrit and >5% parasitemia
^{125}I-Na 1 mCi/10 μL (Amersham IMS.30)
PBS (pH 7.2) (stored at 4 °C)
lactoperoxidase, 2 mg/mL (Sigma L8257) (stored at −20 °C)
30% H_2O_2 (stored at 4 °C)

Labeling buffer:
100 mL PBS (pH 7.2)
0.087 g K_2HPO_4
0.1 mL 1 mM KI

washing buffer:
100 mL PBS (pH 7.2)
0.83 g KI

Procedure (On ice at all times)

- Use cells from a culture of 10 to 20% parasitemia (not lower than 5% at any case) with a majority of trophozoite stages.
- Wash the cells 3 times with PBS, then transfer 200 μL of the washed packed PRBC into a fresh centrifuge tube and add 800 μL of Labeling buffer.
- Add 0.5 to 1 mCi of ^{125}I-Na to the cell suspension.
- Add 100 μL of lactoperoxidase stock solution (2 mg/mL).
- Dilute the stock solution of H_2O_2 1,000-fold in PBS just before use.
- Add 25 μL of the diluted H_2O_2 to the cells; mix gently.
- Wait 1 min and repeat the addition 3 times (4 times altogether).
- At 1 min after the last addition of diluted H_2O_2, wash the cells 4 times in ice-cold washing buffer.
- The cells are now ready for separation/enrichment of mature stages in Percoll/sorbitol gradients as described in PARASITES, section IV:D.

Reference

Fernandez V, Treutiger CJ, Nash GB, Wahlgren M. 1998. Multiple adhesive phenotypes linked to rosetting binding of erythrocytes in *Plasmodium falciparum* malaria. Infect Immun 66(6):2969-2975.

I:B. Solubilization/extraction of surface-radiolabelled proteins

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Equipment

microfuge, refrigerated

Materials and reagents

Solution containing:

1% Triton X-100
1 mM PMSF
1 mM EDTA
1 μM leupeptin
2 μM pepstatin
1% ε-amino-*n*-caproic acid in PBS

SDS–PAGE sample buffer:

2% SDS
5% 2-mercaptoethanol

Procedure

- Use the pellet of labelled PRBC enriched for mature stages on Percoll/sorbitol gradient as described above (see PARASITES, section IV:E).
- Resuspend the pellet in 0.25 to 0.5 mL of a solution containing 1% Triton X-100, 1 mM PMSF, 1 mM EDTA, 1 μM leupeptin, 2 μM pepstatin, and 1% ε-amino-*n*-caproic acid in PBS. Incubate the pellet on ice for 1 h with periodic vortexing.
- Centrifuge the samples in a microfuge at 4 °C,at maximum speed for 15 min. Separate and save the supernatant (the fraction soluble in Triton X-100).
- Resuspend/extract the pellet in SDS–PAGE sample buffer, mix well by pipetting, boil for 4 min, mix again, and centrifuge it for 3 min at maximum speed in a microfuge.
- Separate the supernatant (the fraction soluble in SDS).
- Run aliquots of Triton X-100 and SDS-soluble fractions on SDS–PAGE gels (6% homogeneous or 5 to 8.5% gradients for proteins above Band 3; i.e., M.W. ~100,000, 7.5 to 17.5% gradients for proteins below Band 3).

Reference

Fernandez V, Treutiger CJ, Nash GB, Wahlgren M. 1998. Multiple adhesive phenotypes linked to rosetting binding of erythrocytes in *Plasmodium falciparum* malaria. Infect Immun 66(6):2969-2975.

I:C. Alternative solubilization protocol

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Equipment

SDS–PAGE gel apparatus
microfuge

Materials and reagents

2% SDS
PBS containing 0.1 mg/mL of leupeptin and 0.1 mg/mL of chymostatin protease inhibitors
PBS containing 1% Triton X-100, 1 mM PMSF and 1% ε-amino-*n*-caproic acid
protein A–Sepharose (Amersham Biosciences 17-0780-01 or Sigma)

Preparation

- Solubilize ~200 μL of erythrocytes (the pellet from one flask) in 4 to 8 mL of 2% SDS (dissolved in PBS containing 0.1 mg/mL of leupeptin and 0.1 mg/mL of chymostatin protease inhibitors).
- Incubate the sample at room temperature for 30 to 90 min. Any possible background radiation from the protein A–Sepharose can be adsorbed by mixing washed protein A–Sepharose with the solubilized sample and letting it incubate for 1 h prior to the immunoprecipitation.

Solubilization

- Extract surface-radioiodinated proteins by using 0.2 mL of pellet plus 1.8 mL of PBS containing 1% Triton X-100, 1 mM PMSF, and 1% ε-amino-*n*-caproic acid and incubating the tube at 4 °C on ice for 1 h.
- Centrifuge the extract in a microfuge at maximum speed for 15 min. Remove the Triton X-100 soluble supernatant, then freeze the supernatant at −70 °C.
- Extract the insoluble pellet in SDS–PAGE sample preparation buffer.
- Separate the proteins on a 7.5 to 17.5% linear gradient SDS–PAGE gel.

Reference

Mattei D, Scherf A. 1992. The Pf332 gene of *Plasmodium falciparum* codes for a giant protein that is translocated from the parasite to the membrane of infected erythrocytes. Gene 110(1):71-79.

I:D. Immunoprecipitation of ^{125}I-labelled PfEMP1

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Equipment

microcentrifuge
PhosphorImager

Materials and reagents

^{125}I-labelled SDS extract, preferably use 100,000 or more cpm per sample
^{125}I-labelled Triton X-100, if desired
2% Triton X-100 in PBS
hybridoma supernatant to which has been added 25 mM of HEPES (pH 7.4), 0.5% Triton X-100, 5 mM EDTA, and protease inhibitors
Protein A-sepharose (Amersham Biosciences 17-0780-01)

NETT buffer:
150 mM NaCl, MW 58.44 (8.77 g/liter)
5 mM EDTA, MW 380.2 (1.9 g/liter)
50 mM Tris-base, MW 121.1 (6.06 g/liter)
0.02% sodium azide
0.5% Triton X-100
Adjust to pH 8.1 if mouse antibody is used, otherwise use pH 7.4.

NETT with final 0.3 M NaCl
NETT with 10 mg/mL Ig-free BSA containing protease inhibitors (1 mM PMSF, 1 mM EDTA, 1 mM leupeptin, 2 mM pepstatin, and 1% ε-amino-*n*-caproic acid in PBS)
NETT with 10 mg/mL BSA Fraction V
secondary antibodies as appropriate
protein A–Sepharose equilibrated with NETT with Ig-free BSA without protease inhibitors, make up as a 1:1 suspension (i.e., 50 μL of Sepharose gel : 50 μL of suspension)
5% SDS Sample Buffer (= 2× concentration)

Procedure

Day 1

- Reconstitute SDS extract by adding an equal volume of 2% Triton X-100 in PBS and 20 volumes of NETT plus Ig-free BSA containing protease inhibitors.
- Aliquot the reconstituted SDS extract in microfuge tubes allowing about 100,000 cpm per tube (or more if it is available).
- Add primary antibody:
  For sera, use 10 μL/sample for monoclonal antibodies (you can go up to 20 μL with human or *Aotus* sera if desired), but rabbit sera generate a high MW aggregate which messes up this region on the gel. Use 2 to 5 μL of hybridoma supernatant to which has been added 25 mM of HEPES (pH 7.4), 0.5% Triton X-100, 5 mM EDTA, and protease inhibitors.
- Incubate the sample overnight at 4 °C with slow rotation/mixing.

Day 2

- Add secondary antibodies if using primary antibodies not binding protein A. Leave the tubes for 1 h at room temperature.
- Add 100 μL of the above equilibrated protein A–Sepharose.
- Leave it at room temperature for 1 h with intermittent mixing.
- Take off the supernatant and put in ^{125}I liquid waste. Wash the gel–slurry with the following solutions and quick, ~30-s spins in a microcentrifuge on the high setting:
  - 1 mL NETT–BSA
  - 1 mL NETT
  - 1 mL NETT with 0.3 M NaCl
- Leave the slurry for 20 min to allow NaCl to equilibrate into the beads. Then wash again:
  - 1 mL NETT with 0.3 M NaCl
  - 1 mL NETT
- Take off all supernatant.
- Add 50 μL of 2× Sample Buffer to the pellet. Mix and freeze at −70 °C until use in SDS–PAGE gel electrophoresis.

Notes on the capacity of protein A–Sepharose

According to the manufacturer's specification, the capacity is 20 mg of human-IgG per milliliter of gel or 3 to 10 mg of mouse-IgG per milliliter of gel. Taking 10 mg/mL as above, then 50 μL of gel used for immunoprecipitation has a maximum capacity of 500 μg of IgG. Based on the principle that it is best to use less than 20% of capacity to get complete binding, one should aim to use maximally 100 μg of IgG.

e.g., 10 μL of rat serum = 100 μg of IgG. Add 50 to 100 μg of secondary antibody IgG.

Autoradiography for the measurement of γ rays

Stain, destain, and dry the SDS–PAGE gel. Expose a PhosphorImager screen (or an X-ray film using an intensifying screen) at room temperature.

Reference

van Schravendijk MR, Rock EP, Marsh K, Ito Y, Aikawa M, Neequaye J, Ofori-Adjei D, Rodriguez R, Patarroyo ME, Howard RJ. 1991. Characterization and localization of *Plasmodium falciparum* surface antigens on infected erythrocytes from West African patients. Blood 78(1):226-236.

I:E. Surface biotinylation of infected erythrocytes

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Equipment

centrifuge (10.000 × g), refrigerated
appropriate reaction tubes for centrifuge

Materials and reagents

sulfosuccinimidyl-6-(biotinamido) hexanoate (sulfo-NHS-LC-biotin; Pierce Chemicals). Prepare fresh each time.
inhibitors of "*New permeation pathways"*:
Furosemide (Sigma) or 5-nitro-2-(3-phenylpropylamino)-benzoic acid (NPPB; Sigma). Prepare stock solution (10 mM in DMSO)
washing buffer (PBS 2+): PBS containing 0.6mM $CaCl_2$, 1mM $MgCl_2$, pH 7.6 and PIC (1:500)
biotinylation buffer: 1 mg/mL sulfo-NHS-LC-biotin in PBS 2+
blocking buffer: PBS 2+ containing 100 mM glycine
buffers contain:
protease Inhibitor Cocktail Set III (Calbiochem), dilution 1:500

Procedure

- prior to biotinylation wash 10^8 infected erythrocytes three times in PBS 2+
- incubate cells in PBS 2+ containing 1 mg/mL sulfo-NHS-LC-biotin and 100 µM of furosemide or NPPB for 30 min on ice.
- sediment cells by centrifugation at 10,000 x g for 15 s at 4 °C. (analyze supernatant photometrically at 570 nm for the release of haemoglobin)
- to block and remove unbound biotin, wash cells three times in blocking buffer
- cells are now ready for further analyses.

Comments

Late schizonts tend to disintegrate during the process leading to lysis and internal biotinylation.

References

Nyalwidhe, J., Baumeister, S., Hibbs, A.R., Tawill, S., Papakrivos, J., Volker, U., and Lingelbach, K. (2002) A nonpermeant biotin derivative gains access to the parasitophorous vacuole in *Plasmodium falciparum* infected erythrocytes permeabilized with streptolysin O. J Biol Chem 277: 40005–40011.

Baumeister, S., Endermann, T., Charpian, S., Nyalwidhe, J.,Duranton, C., Huber, S., Lang, F., Kirk, K and Lingelbach, K. (2003) A biotin derivative blocks parasite induced novel permeation pathways in *Plasmodium falciparum*-infected erythrocytes. Mol Biochem Parasitol 132: 35–45.

II. Studies of the nuclear components of *Plasmodium falciparum*

II.A. Chromatin Immunoprecipitation (ChIP) Assay

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Equipment

centrifuge 4 ºC
microcentrifuge (4ºC and room temperature)
Douncer homogenizer
light Microscope
Bioruptor UCD-200 (Diagenode)

Materials and reagents

malaria culture with 6-8 % parasitemia
PBS
saponin
3M sodium acetate pH 5.2
glycogen
protease inhibitors
absolute Ethanol
70% Ethanol
Proteinase K
phenol:Chloroform:isoamyl alcohol
SDS 10%
$NaHCO_3$ 1M
Salmon Sperm DNA/protein A agarose slurry 50%
formaldehyde 37%
1.25M glycine
Nonidet-P40 10%

Buffers

Lysis Buffer:
10 mM Hepes pH 7.9
10 mM KCl
0.1 mM EDTA pH 8:0
0.1 mM EGTA pH 8.0
1 mM DTT (add just before using)
The Lysis Buffer is supplemented with protease inhibitors

SDS Lysis Buffer
1% SDS
10 mM EDTA
50 mM Tris-HCl pH 8.1

ChIP Dilution Buffer
0.01% SDS
1.1% Triton X-100

1.2 mM EDTA
16.7 mM Tris-HCl pH 8.1
150 mM NaCl

Low Salt Immune Complex Wash Buffer
0.1% SDS
1% Triton X-100
2 mM EDTA
20 mM Tris-HCl pH 8.1
150 mM NaCl

High Salt Immune Complex Wash Buffer
0.1% SDS
1% Triton X-100
2 mM EDTA
20 mM Tris-HCl pH 8.1
500 mM NaCl

LiCl Immune Complex Wash Buffer
0.25 M LiCl
1% NP-40
1% Deoxycolate
1mM EDTA
10 mM Tris-HCl pH 8.1

TE Buffer
10 mM Tris-HCl pH 8.0
1 mM EDTA pH 8.0

Elution Buffer
1% SDS
0.1 M $NaHCO_3$
To be prepared before using

SAMPLE
6 x 10^9 rings (~8 immunoprecipitations)
2 x 10^9 trophozoites (~8 immunoprecipitations)
1 x 10^9 schizonts (~12 immunoprecipitations)
(~15 μg of chromatin per immunoprecipitation)

Procedure

Preparation of cross-linked chromatin

- Add 37% formaldehyde to the cultures to get a final concentration of 1%. Mix immediately and incubate at 37 ºC with agitation for 5 min.
- Note: Cross-linking time influences the efficiency of chromatin shearing and the efficiency of precipitating a specific antigen. For histone modifications 5 min is sufficient. For transcription factors longer cross-linking times may be necessary.

- To stop crosslinking add the amount of 1.25 M Glycine needed to achieve a final concentration of 0.125 M. Place the flask in ice and agitate for 5 min.
- Wash sample 3 times with cold PBS (centrifugations should be carried out at 4ºC) Note: The formaldehyde may cause some red blood cell lysis.
- Add saponine so that the final concentration is 0.06%. Incubate for 5-10 min (until complete RBC lysis). If RBC lysis is not complete, add more saponine until the final concentration is 0.15%.
- Spin the sample (4000 rpm 4 ºC 10 min) and wash the pellet with cold PBS until the supernatant becomes clear.
- Parasite pellets can be stored at -80 ºC.
- Prepare nuclei by resuspending the crosslinked parasites in Cold Lysis Buffer:
  for ~6 x 10^9 rings: 2 mL of Cold Lysis Buffer
  for ~2 x 10^9 trophozoites: 2 mL of Cold Lysis Buffer
  for ~1 x 10^9 schizonts: 3 mL of Cold Lysis Buffer.

- Transfer to a pre-chilled douncer homogenizer and set on ice for 30 min. Add 10% Nonidet-P40 to reach a final concentration of 0.25%. Lyse the parasite with:
  200 strokes for ~6 x 10^9 rings/2 mL of Cold Lysis Buffer
  100 strokes for ~2 x 10^9 trophozoites/2 mL of Cold Lysis Buffer
  100 strokes for ~1 x 10^9 schizonts/3 mL of Cold Lysis Buffer

- Check for parasite lysis with the help of a light microscope.
- Centrifuge the lysate for 10 min at 14000 rpm 4 ºC.
- Resuspend the pellet in:
  400 μL of SDS Lysis Buffer for ~6 x 10^9 rings
  400 μL of SDS Lysis Buffer for ~2 x 10^9 trophozoites
  600 μL of SDS Lysis Buffer for ~1 x 10^9 schizonts.

- Note: 1% SDS improves the efficiency of sonication (next step) but could negatively affect the recovery for some antibodies. Performing the sonication in a 0.1% SDS-containing buffer compromises shearing efficiency; therefore, sonication conditions should be carefully controlled. Another option is to use SDS Lysis Buffer containing 1% SDS, followed by dialysis against the same buffer with a lower SDS concentration.

Chromatin sonication

- A "pre-cooling" of the Bioruptor´s tank with crushed ice 30 min before starting is recommended to avoid water heating too quickly.
- Bioruptor settings: Power: High, Cycling parameter: 30 sec ON 30 sec OFF.
- Sonicate for 8 min (200 μL is the maximum volume per 1.5 mL tube).
- Replace the water with cold water and crushed ice.
- Sonicate for 8 min.
- Remove debris by centrifuging for 10 min at 12500 g 4ºC 10 min.
- Dilute supernatant fraction 10 fold in ChIP Dilution Buffer. Add protease inhibitors. Keep a portion of this chromatin solution (20 μL) as DNA input and another portion (80 μL) to check chromatin shearing. These two portions will be processed with the other immunoprecipitations during DNA purification.

Chromatin immunoprecipitation

- To reduce non-specific background, pre-clear the chromatin solution with 50 μL/mL of salmon sperm DNA/protein A agarose slurry for 2 hours at 4 ºC with agitation. Pellet agarose by brief centrifugation and transfer the supernatant to a new tube.
- Make aliquots of the chromatin solution (500 μL/aliquot).
- Add the antibody (the concentration of the antibody should be empirically determined) and incubate overnight at 4 ºC with rotation.
- To collect immune complexes, incubate the sample with 50 μL per sample of Salmon sperm DNA/protein A agarose slurry for 2 hours at 4 ºC with agitation.
- Pellet beads by gentle centrifugation (1000 g x 1 min). Carefully remove the supernatant that contains unbound chromatin.
- Wash the protein A agarose/antibody/chromatin complex for 5 min on a rotating platform with 1 mL of each of the buffers listed below. Discard the wash buffer between steps:

  Low Salt Immune Complex Wash Buffer (at 4 ºC)
  High Salt Immune Complex Wash Buffer (at 4 ºC)
  LiCl Immune Complex Wash Buffer (at 4 ºC)
  TE buffer, two washes at room temperature

- Elute immune complexes by adding 100 μL of fresh Elution Buffer. Vortex briefly to mix, and incubate at room temperature for 15 min with rotation. Spin down beads, carefully transfer the supernatant fraction (eluate) to another tube and repeat elution with 150 μL of fresh Elution Buffer. Combine eluates.

DNA purification

- To reverse cross-linking, incubate the immunoprecipitations and the input at 65ºC for 6 hours.
- Proteinase K treatment. Add 2.5 μL of 20 mg/mL Proteinase K and incubate for 2 hours at 45ºC.
- Phenol/chloroform/isoamylic alcohol extraction.
- Ethanol precipitation.
- Resuspend the pellet in 300 μL of distilled water.
- Quantify immunoprecipitated DNA by dot blot or semiquantitative or quantitative PCR. In this last case, use 5 μL of sample in a 20 μL PCR reaction.

II.B. Immunolocazation of Nuclear Antigens in *Plasmodium falciparum*

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Equipment

Waterbath at 37 °C
Microscope

Materials and reagents

Saponin (Sigma)
RPMI 1640 (Gibco)
Microscope slides (Cell-Line) and Coverslips
10% Paraformaldehyde (Electron Microscopy Sciences)
Bovine serum albumin (BSA) (Sigma)
VECTASHIELD mounting medium with DAPI (Vector Laboratories)
Nail polish

Procedure

Parasite fixation

- Treat the parasites (~2,5% parasitemia) with Saponin (20min for ring stages and 5min for mature stages), then centrifuge 4000rpm for 5min
- Wash twice in RPMI (centrifuge 6000rpm 1min)
- Resuspend the parasites in Paraformaldehyde 4% (in PBS) for 10-15min, on ice
- Wash once with cold PBS (centrifuge 6000rpm 1min)
- Resuspend in 100µL cold PBS (fixed parasites can be stored for at least a week at 4°C prior to IF analysis)

Antibodies*:*

- Dilute the primary antibody in 100µL PBS-BSA 1%
- Centrifuge the fixed parasites, resuspend the pellet in the diluted primary antibody and incubate for 30-45 min at 37°C
- Wash twice in PBS (centrifuge 6000rpm)
- Resuspend the pellet in the diluted secondary antibody conjugated with fluorochrome (in PBS-BSA 1%) and incubate for 30 min at 37°C (protected from light)
- Wash twice in PBS (centrifuge 6000rpm)
- Resuspend the pellet in PBS and deposit on microscope slides
- Let air dry the slide and mount using Vectashield with DAPI
- Analyze the slide by fluorescence microscopy
- If z-stack analysis will be performed the coverslips must be previously coated with poly-lysine or poly-ethylenimine 1%

Comments

All the steps are performed in suspension, so the nuclear architecture is better preserved.
The saponin lysis does not allow analysis of erythrocyte-surface antigens, but produces preparations with very reduce background.

II.C. Extraction and purification of *P.falciparum* Histones

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Equipment

microcentrifuge (4°C)
vortex

Materials and reagents

malaria culture of 6-8% parasitemia, enriched for mature stages (preferably tophozoites, 30-40 hours post invasion).
PBS 1x (Ice cold)
saponin
hemoglobin Removal Buffer
- 25 mM Tris-HCl (pH 7.8)
- 1 mM EDTA
- 0.2% (v/v Nonidet P40)

Distilled H_2O
0.8 M NaCl
0.25 M HCl
20% TCA (Tri-chloro acetic acid, stored at 4 °C)
acetone (stored at -20°C)
SDS PAGE Sample Buffer

Procedure

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- Pellet 2-3 mL of healthy malaria culture with at least 6-8% parasitemia and lyse with saponin
- Resuspend pellet with 1.5 vol. PBS and add saponin to obtain a final concentration of 0.06%.
- Mix gently and incubate on ice for 5-10 min.
- Add 5 vol. of PBS and centrifuge at 3000 rpm for 5 min.
- Discard the supernatant and transfer the pellet to a 1.5 mL microcentrifuge tube
- Wash the resulting pellet at least 3 times (6000g for 2 min each) with ice cold PBS till the supernatant is clear of visible haemoglobin.
- Resuspend the resulting pellet in 2 vol. of haemoglobin removal buffer to further remove contaminating membranes and haemoglobin.
- Centrifuge briefly at 12000g for 30 s.
- Wash the resulting pellet twice (till the supernatant is clear) in the same buffer 12000g for 30 s.
- Wash 1 time with ice cold distilled H_2O (12000g for 30 s).
- Wash the pellet 2 times in 0.8 M NaCl. (Care should be taken while removing the supernatant subsequent to each wash as the pellet is viscous and gooey and may quickly expand into the supernatant or sucked up with it. It may be advisable to draw up minimal amount of supernatant and the gooey pellet and transfer to a new eppendorf tube after each wash to avoid sucking up and disintegrating the pellet each time).

- Acid extract the resulting pellet by adding 8 vol of 0.25 M HCl followed by vigorous vortexing and subsequent incubation at 4 °C for 2 hours to overnight.
- Recover the acid soluble proteins in the supernatant by centrifuging at 12000g for 30 minutes. The pellet contains acid insoluble contaminants.
- To the supernatant mix equal volume of 20% TCA, mix by inverting and incubate on ice for 15 minutes.
- Centrifuge at 12000g for 15 minutes. (At the end of the spin, immediately place the samples on ice. As the samples are removed from the microfuge, a minisicule separate phase may appear at the bottom of the tube.This may be clear or cloudy, depending on which care should be taken in the next step while removing the supernatant).
- Carefully remove the supernatant. (If the pellet is not visible, place the pipett tip in the centre of the tube but not touching the bottom or the sides of the eppendorf while removing the supernatant).
- Add 500 μL of acetone to each sample, flick the tube gently and incubate at -20 °C for at least 1 hour. (After adding acetone to each sample, care should be taken to keep everything in the lower half of the tube as the protein precipitates as a thin chip or aggregate and may easily be lost somewhere on the lid of the tube).
- Centrifuge at 12000g for 15 minutes.
- Carefully remove the supernatant and set tubes on their sides at RT for 5-10 minutes to dry the pellet and evaporate the acetone.
- Dissolve the protein precipitate in SDS PAGE Sample buffer and analyse on 15% SDS Polyacrylamide gels. Individual histones should separate out in 5 distinct bands as in example coomassie stained extract in Fig. 1.

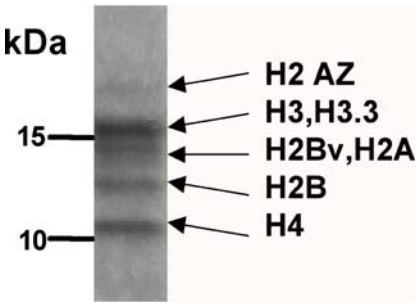

Fig. 1

II.D. Novel Histone Extraction Protocol retaining maximal posttranslational modifications

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To study posttranslational histone modifiations, it is of upmost importancce to use extraction protocols that maintain labile modifications such as phosphorylation. The presented protocol has been optimized for the detection of phosphorylation, acetylation, and methylation.

Equipment

Microcentrifuge @ 4°C
Bioruptor UCD-200 (Diagenode) @ 4°C

Materials and reagents

Malaria culture @ 5% parasitemia
PBS @ 4°C
Saponin
Complete protease inhibitor (PI) [Roche, 11697498001]
Complete phosphatase inhibitor (PPI) [Roche, 4906845001]
NaCl stock solution [2M]
Active Motif histone purification mini kit [cat. no. 40026]
Ultracel-3K centrifugal filter units [Millipore, UFC800308]
12% SDS-PAGE gel [Bio-Rad, 345-0118]
MES running buffer [Invitrogen, NP0002]
Bio-safe coomassie blue [Bio-Rad, 161-0786]

Buffers

No-salt buffer (2mM EDTA, 0.1mM EGTA)
Low salt buffer (200mM NaCl, 2mM EDTA, 1mM DTT)
Equilibration buffer (50mM Tris-HCl, pH 8.0, 200mM NaCl, 2mM EDTA)
Wash buffer (50mM Tris-HCl, pH 8.0, 500mM NaCl, 2mM EDTA)
Elution buffer (50mM Tris-HCl, pH 8.0, 2M NaCl, 2mM EDTA)

Experimental Procedure

Histone Extraction: Histones were obtained by acid extraction and high-salt extraction techniques from unsynchronized culture of wild type 3D7 parasites. Parasites for this purpose were grown in human blood that had been washed to deplete white blood cell contamination. Complete protease inhibitor (PI) and complete phosphatase inhibitor (PPI) were used during all steps, starting from collection of infected red blood cells (iRBC) till extraction of histone samples. All steps were performed at 4°C to minimize enzymatic activities that could potentially interfere with PTMs. For both types of extraction, 6 ml of iRBC of 5% parasitemia were collected and were lysed on ice using 0.15% saponin. The parasites were then washed three times in ice cold PBS until the supernatant was clear and no blood was observed in the parasite pellet. The resulting pellet was further treated with 0.06% saponin to remove any leftover blood contamination and washed three more times in ice cold PBS. The resulting parasite pellet was differentially treated as follows for acid and high salt extraction methods respectively.

Acid Extraction of Histones: Histones were acid extracted using Active Motif histone purification mini kit, following manufacturer's recommendation, with slight modifications. Briefly, the parasite pellet was resuspended in 10 ml of ice cold Extraction Buffer and sonicated for 5 minutes (30 seconds ON/OFF cycle) at 4°C using Bioruptor UCD-200. The pellets were incubated overnight at 4°C to extract total histones. Cellular debris was removed by spinning at 16000xg at 4°C for 10 minutes.

High-salt Extraction of Histones: The parasite pellet was resuspended in 10 ml of no-salt buffer and sonicated for 5 minutes (30 seconds ON/OFF cycle) at 4°C using Bioruptor UCD-200. To this solution, NaCl was added to a final concentration of 2M and incubated overnight at 4°C. Cellular debris was pelleted by spinning at 16000xg at 4°C for 10 minutes. The supernatant containing crude histones was then buffer exchanged with low salt buffer and concentrated to 1ml volume using Ultracel-3K centrifugal filter units. In the mean time, packed sulfopropyl (SP) resin column supplied with the Active Motif histone purification mini kit was equilibrated with equilibration buffer. The concentrated crude histone extract was passed through this column to bind extracted histones. The column was washed 3 times with wash buffer to wash away contaminating proteins. Total core histones were eluted in elution buffer. The purified histones were run on 12% SDS-PAGE gel with MES buffer to attain good separation of histones and stained with Bio-safe coomassie blue (detection limit 50-100 ng) to check for the purity of the extracted histones.

III. Metabolic Labeling of *Plasmodium falciparum*

III:A. Metabolic Labeling of *Plasmodium falciparum*-infected erythrocytes

by ***Mats Wahlgren***
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Materials and reagents

RPMI custom medium, containing everything including serum, but without methionine
healthy parasites at ring-stage
0.5 mCi of ^{35}S-methionine (Amersham SJ1015)
RPMI 1640

Procedure

- Use ring-form parasites to label, then let them develop into late stages (20 to 30 h) for analysis.
- Use RPMI custom medium, containing everything including serum, but without methionine.
- Use as high a parasitemia as possible (i.e., healthy parasites at ring-stage).
- Use 0.5 mCi of ^{35}S-methionine per small culture flask (25 cm^2).
- Remove the old medium, as usual, leaving a few drops of the old medium in the flask.
- Mix 0.5 mCi of ^{35}S-methionine with 5 mL of medium and add to the flask.
- Incubate the flask as usual at 37 °C for approximately 30 h, until late parasite stages develop.
- Wash the parasites 3 times with RPMI 1640.
- Freeze the parasite pellet at −20 °C.

Comments on autoradiography for measuring β rays

- Stain and destain the gel. If it will be exposed on an X-ray film, use Amplify (Amersham) after the last destaining bath. Use enough Amplify for the gel to float freely and let it soak for 15 to 20 min. This step is not needed if a PhosphorImager screen will be used.
- Dry the gel. Expose the X-ray film to the gel at −70 °C using an intensifying screen, or simply use a PhosphorImager screen at room temperature.

Reference

Helmby H, Cavelier L, Pettersson U, Wahlgren M. 1993. Rosetting *Plasmodium falciparum*-infected erythrocytes express unique strain-specific antigens on their surface. Infect Immun 61(1):284-288.

III:B. Immunoprecipitation using metabolically labelled parasite extracts

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Equipment

immufuge

Materials and reagents

NETT-buffer 1 (NETT-1):
- 150 mM NaCl, MW 58.44 (8.77 g/L)
- 5 mM EDTA, MW 380.2 (1.9 g/L)
- 50 mM Tris-base, MW 121.1 (6.06 g/L)
- 0.02% sodium azide
- 0.5% Triton X-100
- Adjust to pH 8.1 if mouse antibody is used, otherwise use pH 7.4.

NETT-buffer 2 (NETT-2):
- same as above except use 500 mM NaCl (29.22 g/liter).
- Adjust to pH 8.1 if mouse antibody is used, otherwise use pH 7.4.

10% Triton X-100
1% BSA
50% protein A–Sepharose (washed with NETT-buffer 1)
SDS–PAGE sample buffer:
- 100 mM Tris-HCL, pH 6.8
- 10% Glycerol
- 5% SDS
- 2% 2-mercaptoethanol
- bromophenol blue

0.25% Coomassie blue R-250
Amplify (Amersham)

Procedure

- Mix in a conical centrifuge tube:
  - 300 μL NETT-1
  - 100 μL 10% Triton X-100
  - 25 to 100 μL antibodies
  - 100 μL solubilized sample
  - 1% BSA
- Incubate the mix for 90 min at room temperature or 4 °C, slowly rotating/mixing.
- Add 75 μL of 50% protein A–Sepharose. Incubate the mix for 30 to 60 min at room temperature. Shake the tube a little now and then.
- Wash the Sepharose mixture and centrifuge it in a blood centrifuge (Immufuge) for 15 s on low setting:
  - twice with NETT-1
  - once with NETT-2
  - twice with NETT-1

- Add 50 μL of sample prep. buffer and boil the solution for 2 min. Run the sample on SDS–PAGE.
- Stain and destain the gel, use Amplify for no more than 20 min (see IMMUNOCHEMISTRY, section II:A above), then dry the gel at 60 °C.
- Put the dried gel on film, without intensifying screen, in a cassette at −70 °C for at least two weeks or simply use a PhosphorImager screen at room temperature.

Reference

Helmby H, Cavelier L, Pettersson U, Wahlgren M. 1993. Rosetting *Plasmodium falciparum*-infected erythrocytes express unique strain-specific antigens on their surface. Infect Immun 61(1):284-288.

IV. Resolution of giant proteins (200 kDa to 1 MDa) of *Plasmodium falciparum* on polyacrylamide–agarose composite gels

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Equipment

SDS–PAGE gel apparatus

Materials and reagents

polyacrylamide–agarose composite gel:
- 375 mM Tris–HCl (pH 8.8)
- 0.5% agarose
- 3% acrylamide (29:1, acrylamide:bisacrylamide)
- 0.1% SDS

ammonium persulfate
N,N,N′,N′-tetramethyl-ethylenediamine (TEMED)
sample buffer:
- 10% SDS
- 250 mM Tris–HCl (pH 8.8)
- 20% glycerol
- 0.1% bromphenol blue

running buffer:
- 25 mM Tris–HCl (pH 8.3)
- 250 mM glycine
- 0.1% SDS

transfer buffer:
- 48 mM Tris
- 39 mM glycine
- 0.0375% SDS

3% 2-mercaptoethanol (optional)

Preparation

- Prepare the polyacrylamide–agarose composite gel. Dissolve the agarose by boiling it in the Tris buffer, cool it to 50 °C, and then add the acrylamide and SDS.
- Initiate the polymerization by adding ammonium persulfate and TEMED to a final concentration of 0.1% and 1 μL/mL of gel, respectively. Cast the gel in a standard horizontal chamber without a stacking gel layer.
- Boil the protein extracts for 5 min in sample buffer and load them on the gel. The addition of 3% 2-mercaptoethanol to the sample buffer is optional. In the presence of 2-mercaptoethanol, samples are denatured by heating to 50 °C for 15 min.
- Size-fractionate the proteins at 15 V/cm in running buffer for 1 to 2 h.
- Transfer the size-fractionated proteins to a nylon membrane using a semidry transfer device at 3.5 mA/cm^2 in transfer buffer for 2 h.
-

Reference

Wiesner J, Mattei D, Scherf A, Lanzer M. 1998. Biology of giant proteins of *Plasmodium*: resolution on polyacrylamide–agarose composite gels. Parasitol Today 14(1):38-40.

V. Recovery of *Plasmodium falciparum* native proteins and active enzymes

V:A. Solubilization in the presence of non-detergent sulphobetaines, mild solubilization agents for protein purification

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Equipment

centrifuge

Materials and reagents

RPMI 1640
10% Triton X-100 (stock solution)
mixed ion-exchange beads AG 501-X8 (Bio-Rad)
3 M NDSB 201 (3-{1-pyridinio}-1-propanesulfonate) (Fluka) dissolved in warm water and repurified on mixed ion-exchange beads); kept at −20 °C
2% Triton X-100, 2 M NDSB 201 (extraction solution; kept at −20 °C)
leupeptin and pepstatin (10 µg/mL) (optional) (stock solutions)
double-distilled water
sulphobetaine SB3-14 (optional)

Preparation

- Wash pelleted PRBC twice in RPMI 1640.
- Resuspend the pellet in 1 volume of double-distilled water.
- Freeze at −20 °C and thaw at room temperature.
- Add 2 volumes of:
  - 2% Triton X-100 (or detergent sulphobetaine SB3-14)
  - 2 M NDSB 201
  - leupeptin and pepstatin (10 µg/mL), if needed
  - **no** Tris buffer (usually 10 mM [pH 7.5]) if followed by isoelectric focusing
- Incubate the pellet for 30 min at room temperature.
- Centrifuge the pellet for 30 min at 15,000 × *g* at 4 °C; keep the supernatant (extract) frozen at −20 °C.

Reference

Vuillard L, Braun-Breton C, Rabilloud T. 1995. Non-detergent sulphobetaines: a new class of mild solubilization agents for protein purification. Biochem J 305(1):337-343.

V:B. Isoelectric focusing of proteins in a Rotofor cell: a first step in protein purification

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The Rotofor cell is a preparative scale, free solution, isoelectric focusing apparatus useful in a protein purification scheme. The cell can achieve 25-fold purification of a protein in a single 4-h run. This technique is especially advantageous when protein activity must be maintained. Bioactivity is maintained because the proteins are in solution in their native conformation.

Equipment

Rotofor cell (Bio-Rad)
pH meter

Materials and reagents

0.1 M NaOH
0.1 M H_3PO_4
1% Triton X-100
1 M NDSB 201 (3-{1-pyridinio}-1-propanesulfonate) (Fluka)
2% ampholytes (Pharmalytes of the appropriate pH range, usually between pH 3 and pH 10, 40% stock solution, Pharmacia)

Preparation

- Equilibrate the ion exchange membranes by overnight incubation in the appropriate electrolyte solution: 0.1 M NaOH for the anion exchange membrane and 0.1 M H_3PO_4 for the cation exchange membrane.
- Cool the Rotofor cell by connecting the cooling finger to a source of circulating coolant.
- Assemble the focusing chamber according to the instruction manual.
- Fill the electrolyte chambers with electrolytes immediately after assembly to prevent the membranes from drying: 25 mL of 0.1 M NaOH for the cathode chamber (–) containing the anion exchange membrane and 25 mL of 0.1 M H_3PO_4 for the anode chamber (+) containing the cation exchange membrane.
- Fill the focusing chamber with 45 mL of focusing solution, using a 50-mL syringe with a needle:
  1% Triton X-100 (or SB3-14; not necessary for soluble proteins)
  1 M NDSB 201
  2% ampholytes
- Prerun the chamber for 1 h at 4 °C, 11 W, 34 mA, and 995 V to preform the pH gradient.
- Load 1 to 5 mL of the sample (protein solution or extract) in 1 M NDSB 201, 2% ampholytes.
- Add the sample in the central channels of the Rotofor focusing chamber to prevent exposure of the proteins to very low or high pH.
- Protein concentration should be adjusted for desired yield. Assuming each component will focus in 1 to 3 fractions; preparative fractionation of 1 to 2 g of proteins has been successfully performed!
- Run the sample for 3 h at 4 °C, 11 W, 34 mA, and 995 V.

- Collect the 20 fractions on ice.
- Measure the pH of each fraction. Take a 100-μL aliquot, add 1 mL of double-distilled water, and keep it at room temperature for 10 min before measuring the pH.
- Store the fractions at −20 °C.

Reference
Roggwiller E, Morales Bétoulle MR, Blisnick T, Braun-Breton C. 1996. A role for erythrocyte band 3 degradation by the parasite gp76 serine protease in the formation of the parasitophorous vacuole during invasion of erythrocytes by *Plasmodium falciparum*. Mol Biochem Parasitol 82(1):13-24.

SEROLOGY

I. Antibody staining of *Plasmodium falciparum*-infected erythrocytes

I:A. Erythrocyte membrane immunofluorescence (EMIF)

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Equipment

centrifuge
humid chamber (wet filter paper in a plastic box)
microscope (UV and light)

Materials and reagents

Plasmodium falciparum cultures in human type O^+ erythrocytes, 5 to 10% parasitemia (see PARASITES, section I:A)
glutaraldehyde (GDA)
biotinylated anti-Ig of the appropriate specificity (Vector Labs)
FITC–Avidine D, F/P 4.9 (Vector Labs).
ethidium bromide (Sigma)
CAUTION: This chemical is toxic and mutagenic. Wear protective clothing and use only in a chemical fume hood.
Tris (Sigma)
Hanks' balanced salt solution (Gibco)
multitest slides (8 wells; ICN Biomedicals)
Pasteur pipettes
micropipettes

TH-buffer (0.15 M Tris-buffered Hanks', pH 7.2):
2.11 g Tris–HCl
0.2 g Tris-base
7.88 g of 0.9% NaCl
Dissolve the ingredients in distilled water and bring volume to 1 liter.
Mix 1 volume of Tris buffer with 1 volume of Hanks' BSS.
Add 0.02% NaN_3 (200 mg NaN_3/1 liter).

coating buffer (pH 9.6):
1.59 g Na_2CO_3
2.93 g $NaHCO_3$
200 mg NaN_3
Dissolve the ingredients in distilled water and bring the volume to 1 liter.

Monolayers

- Wash cultures (5 to 10% parasitemia) in TH and resuspend them to 1% hematocrit.
- Pretreat multitest slides, 8 wells with one drop (~20 μL) per well of coating buffer for 30 min.

- Add one drop per well of the 1% *P. falciparum* culture directly after aspiration of the coating buffer and leave the slides to settle for 30 min.
- Rinse off unbound erythrocytes by gently shaking the slides immersed upside down in TH.

Glutaraldehyde (GDA)-fixation of monolayers

- Quickly but gently cover the wet monolayers with 1 to 2 mL of 1% GDA (in PBS) on top of the slide.
- Decant the GDA after about 10 s and repeat the GDA fixation once.
- Wash the slides with distilled water and air-dry them. Slides can then be stored long-term in a freezer (>10 years).

Staining

All incubations below should be done at room temperature in a humid chamber for 30 min.

- Treat the slides sequentially with one drop (~20 μL) of serial dilutions of sera containing malaria antibodies.
- Use 15 μg/mL of biotinylated anti-Ig of the appropriate specificity followed by 50 μg/mL of FITC–avidin D.
- Wash the slides between the incubations with TH or PBS.
- For counter-staining, use one drop of ethidium bromide solution (10 μg/mL) in each well and rinse the slides with distilled water after a few seconds.

Reference

Perlmann H, Berzins K, Wahlgren M, Carlsson J, Björkman A, Patarroyo ME, Perlmann P. 1984. Antibodies in malarial sera to parasite antigens in the membrane of erythrocytes infected with early asexual stages of *Plasmodium falciparum.* J Exp Med 159(6):1686-1704.

I:B. Erythrocyte membrane staining by enzyme linked antibodies (EMEAS)

by ***Rachanee Udomsangpetch***
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Prepare glutaraldehyde-fixed monolayers as in the protocol for EMIF (see SEROLOGY, section I:A).

Equipment

light microscope with a 100× oil-immersion lens
centrifuge
humid chamber (wet filter paper in a plastic box or dish)

Materials and reagents

Plasmodium falciparum cultures in human type O^+ erythrocytes, 5 to 10% parasitemia (see PARASITES, section I:A)
multitest slides (8 wells; ICN Biomedicals)
Pasteur pipettes
micropipettes
biotinylated anti-Ig (Vector Labs)
streptavidin/ALP (Mabtech)
PBS
ALP-conjugated Substrate Kit (Bio-Rad 170-6432)

coating buffer (pH 9.6):
1.59 g Na_2CO_3
2.93 g $NaHCO_3$,
200 mg NaN_3
Dissolve the ingredients in distilled water and bring the volume to 1 liter.

Staining

All incubations below should be done at room temperature in a humid chamber for 30 min.

- Treat the slides sequentially with serial dilutions of immune sera.
- Use biotinylated anti-Ig of the appropriate specificity and streptavidin/ALP.
- Wash the slides between the incubations with PBS.
- Add freshly prepared substrate.
- Wash slides under distilled water after 20 min, then air-dry them.
- Examine the slides under a light microscope with a 100× oil-immersion lens.

Reference

Udomsangpetch R, Kulane A, Berzins K, Perlmann H, Perlmann P, Wåhlin Flyg B. 1993. Enzyme-linked staining method for light microscopic detection of antibodies to parasite antigens on the membrane of *Plasmodium falciparum* infected erythrocytes. Acta Trop 55(1-2):79-86.

I:C. Parasite immunofluorescence (PARIF)

by ***Rachanee Udomsangpetch***
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This protocol allows for antibody staining of intracellular *Plasmodium falciparum* using air-dried monolayers of infected erythrocytes. For staining of the live, intact PRBC surface, please refer to PARASITES, section IX:B.

Equipment

fan

Materials and reagents

Plasmodium falciparum cultures in human type O^+ erythrocytes, 5 to 10% parasitemia of primarily late stages
PBS

coating buffer (pH 9.6):
1.59 g Na_2CO_3
2.93 g $NaHCO_3$
200 mg NaN_3
Add distilled water up to 1 liter.

Monolayers

- Use *Plasmodium falciparum* cultures in human type O^+ erythrocytes, 5 to 10% parasitemia of primarily late stages.
- Wash cells in PBS and resuspend them to 1% parasitemia.
- Pretreat multitest slides with one drop (10 to 20 μL) per well of a coating buffer for 30 min.
- Aspirate the wells and immediately add one drop of the washed culture to each well.
- Leave it to settle for 30 min.
- Gently aspirate the excess of red cells from each well and air-dry the slides under a fan.
- The slides can be stored in a freezer; dry them well before staining.
- For staining, see the protocol for EMIF in SEROLOGY, section I:A above.

Reference

Udomsangpetch R, Carlsson J, Wåhlin B, Holmquist G, Ozaki LS, Scherf A, Mattei D, Mercereau-Puijalon O, Uni S, Aikawa M, Berzins K, Perlmann P. 1989. Reactivity of the human monoclonal antibody 33G2 with repeated sequences of three distinct *Plasmodium falciparum* antigens. J Immunol 142(10):3620-3626.

I:D. Formaldehyde fixation for immunofluorescence analysis (IFA) of *P. falciparum*

by ***Michael J. Blackman***
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See PARASITES, section IX:C, page 91

II. Antibody selection on immobilized antigen or peptide

*by **Denise Mattei***
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Equipment

PD10 column equilibrated in PBS

Materials and reagents

0.45-μm nitrocellulose membrane
PBS
PBS plus 5% powdered skim milk
3 M $MgCl_2$, 75 mM HEPES/NaOH (pH 7.2), 25% ethylene glycol

Preparation

- Prepare 5 mL of a 5 to 10 μg/mL solution of peptide or protein in PBS.
- Soak a 0.45-μm nitrocellulose-membrane filter in this solution for 30 min at room temperature with gentle agitation, and then soak a second filter in this solution.
- Incubate the filters for saturation in PBS plus 5% powdered skim milk for 90 min at room temperature (or overnight at 4 °C) with gentle agitation.
- Incubate each filter in 5 mL of a 1:20 dilution of appropriate serum in PBS plus 5% powdered skim milk for 60 min at room temperature (or overnight at 4 °C) with gentle agitation.
- Wash the filters in PBS plus 5% powdered skim milk for 30 min at room temperature with gentle agitation.
- Wash the filters twice in PBS.
- Soak the filters in 5 mL of a solution of 3 M $MgCl_2$, 75 mM HEPES/NaOH (pH 7.2), 25% ethylene glycol.
- Incubate the filters for 20 min at room temperature with gentle agitation.
- Recover the solution and load it on a PD10 column equilibrated in PBS for collection of 500-μL fractions.
- Check for the presence of IgG in the fractions by dot blot analysis (5 μL of each fraction).

Reference

Tsang VCW, Wilkins PP. 1991. Optimum dissociating condition for immunoaffinity and preferential isolation of antibodies with high specific activity. J Immunol Methods 138(2): 291-299.

III. ELISA

III:A. Equipment, materials, and reagents

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Equipment

micropipette, multichannel pipette (e.g., Thermo Electron)
microplate washer (e.g., Thermo Electron, Skatron Instruments)
microplate reader (e.g., *V* max kinetic microplate reader with computer program SOFTmax Cat. No. 79-200 105, 79-200 100)
centrifuge, swing out rotor

Materials and reagents

With many proteins or peptides, PBS can be used as a coating solution at least with the above plates; test your system first.

flat-bottomed microtiter plates (Maxisorp from Nunc A/S or High Binding from Costar, Cat. No. 3590)
disposable pipette tips
tubes for preparaions of dilutions (Bio-Rad, Cat. No. 223-9391)
cell culture flasks
RPMI–HEPES (see PARASITES, section I:A)
Hanks' balanced salt solution (Gibco)

PBS 10× stock solution:
40 g $Na_2HPO_4 \cdot 12\ H_2O$
5 g KH_2PO_4
81 g NaCl
1.0 mL 20% NaN_3
Dissolve in distilled water and bring volume to 1 liter.

Coating buffer (pH 9.6):
1.59 g Na_2CO_3
2.93 g $NaHCO_3$
1.0 mL 20% NaN_3
Dissolve the ingredients in distilled water and bring the volume to 1 liter.

Blocking buffer:
0.5% BSA in coating buffer

Tween buffer for dilutions and incubations:
100 mL PBS stock (10×, see above)
5 g BSA
0.5 mL Tween
1.0 mL 20% NaN_3
Dissolve the ingredients in distilled water and bring the volume to 1 liter.

Tween wash:

45 g NaCl
2.5 mL Tween
Dissolve the ingredients in distilled water and bring the volume to 5 liters.

Enzyme substrate buffer:

97 mL diethanolamine
~700 mL distilled water
1 mL 20% NaN_3
Adjust the pH to 9.8 with 1 M HCl (~100 mL), then add 101 mg of $MgCl_2 \cdot H_2O$. Bring buffer volume to 1 liter.

Substrate:

NPP (kept in freezer)
Dissolve 1 tablet of NPP in 5 mL of enzyme substrate buffer; keep the solution in the dark. Incubate plates with the substrate at room temperature and keep them in the dark until you are ready for a reading at OD_{405} (usually in 20 to 40 min).

TH (0.15 M Tris-buffered Hanks', pH 7.2):

2.11 g Tris–HCl
0.2 g Tris-base
7.88 g NaCl
Dissolve the ingredients in distilled water and bring the volume to 1 liter. Add 1.0 mL of 20% NaN_3. Mix 1 volume of Tris buffer with 1 volume of Hanks' balanced salt solution.

Sera:

For antibody determinations, sera may be kept in the refrigerator diluted 1:10 in Tris-buffered Hanks' with 0.02% NaN_3 for many months.

Conjugate:

ALP-conjugated or biotinylated anti-Ig of appropriate specificity at recommended concentration
Streptavidine–ALP (Mabtech) when biotinylated antibody has been used.

60% Percoll solution:

60 mL Percoll
7 mL 10× PBS
33 mL PBS
0.3 M NH_4OH

III:B. Antigens for coating

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Crude parasite antigen

Late stage-infected erythrocytes enriched by gradient centrifugation. For 40 tubes, 80-mL of culture:

- Prepare fresh 60% Percoll solutions before each use. Keep all solutions on ice throughout the preparation.
- Wash a late stage *Plasmodium falciparum* culture once in RPMI–HEPES and resuspend it to 10% hematocrit in cold RPMI–HEPES.
- Distribute in centrifuge tubes, 2 mL/tube.
- Using a Pasteur pipette, gently add 2.5 mL of cold 60% Percoll to the bottom of each tube.
- Centrifuge the tubes in a swing-out rotor at 1,500 × *g* and 4 °C for 15 min (2,000 rpm in Sorvall or Beckman centrifuge).
- Withdraw the cells at the interphases with care, pool them, and wash them 3 times with cold PBS (centrifuging each time for 7 to 8 min at 2,000 rpm). The interphases contain trophozoites and schizonts, while rings and uninfected erythrocytes are found in the pellet.
- Freeze-thaw or sonicate the isolated interphases, determine protein after centrifugation, and coat the plate at 10 μg/mL in coating buffer.
- Contaminating HGG in this antigen is avoided by using Albumax to replace serum in the medium for the parasite cultures (see PARASITES, section I:A).

  Comment: 12 medium size cultivation flasks (75 cm^2, 20 mL of culture) with late stages of 10% parasitemia give about 1.5 mg of protein in the extract.

Peptide/BSA conjugation

- Dissolve 1 mg of peptide in 0.1 mL of distilled water.
- Add 0.3 M NH_4OH, if necessary, until the peptide is dissolved, then add PBS to a total volume of 0.5 mL.
- Dissolve 0.5 mg of BSA in the peptide solution.
- Add 0.5 mL of 0.25% glutaraldehyde in PBS dropwise while stirring.
- Mix the solution gently on a roller drum in the cold, overnight.
- Dialyse against PBS, then store at −20 °C. We use 10 μg/mL for coating.

  Comment: Several malaria peptides are commercially available from Bachem. Bigger peptides as MAPs can be used for coating without BSA conjugation.

III:C. ELISA procedure

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Procedure

- Coat the plate with 50 μL of peptide solution or crude parasite antigen at 10 μg/mL in coating buffer.
- Keep the plate at 4 °C overnight. With many proteins or peptides, PBS can be used as a coating solution. Test your system first.
- Block with 100 μL of 0.5% BSA in coating buffer for 3 to 4 h at 37 °C.
- Wash 4 times with 0.9% NaCl plus 0.05% Tween.
- Add 50 μL of serum samples diluted 1:1000; leave them for 1 h at 37 °C.
- Wash them 4 times with 0.9% NaCl plus 0.05% Tween.
- Add 50 μL of ALP-conjugated or biotinylated anti-Ig of appropriate specificity at the recommended concentration in Tween-buffer; leave for 1 h at 37 °C.
- Wash the sample 4 times with 0.9% NaCl plus 0.05% Tween.
- If biotinylated antibody has been used, add 50 μL of streptavidin–ALP diluted 1:2000 in Tween-buffer; leave the sample for 1 h at 37 °C.
- Wash the sample 4 times with 0.9% NaCl plus 0.05% Tween.
- Develop the sample with 50 μL of NPP (1 tablet/5 mL of substrate buffer) (see SEROLOGY, section III:A) under Materials and reagents and read at OD_{405}.

References

Paulie S, Perlmann P, Perlmann H. 2003. Enzyme-linked immunosorbent assay. *In:* Celis JE, ed. Cell biology: a laboratory handbook. 3rd edition. San Diego: Elsevier Science.

Perlmann H, Perlmann P, Berzins K, Wåhlin B, Troye-Blomberg M, Hagstedt M, Andersson I, Högh B, Petersen E, Björkman A. 1989. Dissection of the human antibody response to the malaria antigen Pf155/RESA into epitope specific components. Immunol Rev 112:115-132.

Rougon G, Ceard B, Van Rietschoten J, Jordan B, Barbet J. 1984. Induction with a synthetic peptide of antibodies to HLA class I C-terminal intracytoplasmic region. Mol Immunol 21(6):461-468.

Wahlgren M, Berzins K, Perlmann P, Björkman A. 1983. Characterization of the humoral immune response in *Plasmodium falciparum* malaria. I. Estimation of antibodies to *P. falciparum* or human erythrocytes by means of microELISA. Clin Exp Immunol 54(1): 127-134

IV. Antibody affinity measurements based on Surface Plasmon Resonance

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A Biacore machine is used together with CM5 sensor chips for binding of proteins (such as recombinant proteins) to a surface by amine coupling, using EDC/NHS chemistry. Antibodies/plasma/other proteins are added in the flow over the chip.

Materials:
CM5 sensor chips
Plastic Vials - 15 mm/4.0 ml polypropylene vials
Plastic Vials - 7 mm/0.8 ml rounded polypropylene micro vials
Caps - 7 mm thin polyethylene snap caps
If the machine has a plate holder
Micro plate- polystyrene 96 well micro plates
Micro plate Foil - self-adhesive transparent plastic foils for 96 well plates
1.0 liter bottle for holding buffers
1.0 liter bottle for holding waste

Reagents: All buffers should be degassed and filteredusing 0.45 μm filters.

HBS-EP buffer, pH 7.4
10 mM HEPES, 150 mMNaCl, 3 mM EDTA, 0.005% Tween20.

0.1 M NHS (N-hydroxysuccinimide)
Dissolve 115 mg of NHS in 10 mL of distilled water. Make 200 μL aliquots and store at -20°C

0.4 M EDC (1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride)
Dissolve 767.0 mg of EDC in 10 mL of distilled water. Make 200 μL aliquots and store at -20°C

1 M Ethanolamine
Mix 1.21 mL of stock ethanolamine (16.6M pH 8.5) with 18.79 mL distilled water.Make 200 uμLaliquots and store at 4°C

10 mM glycine-HCl (pH 1.5)

Coating buffer
0.01 M sodium acetate, pH 4.0
0.01 M sodium acetate buffer, pH 4.0

NSB Reducer
Reduces non-specific binding to dextran sensor surfaces.Carboxymethyl dextran sodium salt (10 mg/ml) in 0.15 M NaCl containing 0.02% sodium azide (NaN3). Store at room temperature.

Coupling of recombinant proteins to SPR chips

- All steps are carried out in a continuous flow of HBS-EP running buffer at 5 µL/min.
- The carboxymethylated dextran surface of the chip is activated using an injection pulse (7 min, 5 µL/min) containing a 50:50 mixture of 0.1 M NHS and 0.4 M EDC.
- The protein immobilization is accomplished by manual injection of the protein solution at 100 µg/mL in coating buffer until the desired response units (~800-1000 RU) are achieved.
- The remaining sites on the sensor surface are blocked by injecting ethanolamine for 7-10 minutes.

Binding assays and analysis

- The SPR binding assays are performed with a constant flow rate of 15 - 30µL/min.
- Plasma/sera samples or monoclonal antibodies are prepared in different dilutions, 1:7.5, 1:15, 1:30, 1:60 or 1:90 (more dilution for samples with high concentrations of antibodies) using a final concentration of 0.5 mg/mL NSB reducer in HBS-EP buffer, pH 7.4.
- An internal positive control sample (plasma or monoclonal antibodies) that can bind with different proteins on the chip must be used after every 10-15 runs. This will help in assessing the quality/stability of the proteins on the chip surface.
- At all dilutions, the associations of antibodies with the immobilized proteins are monitored for 3-6 minutes followed by 10 minutes of dissociation.
- Residual bound antibody is removed by washing the chip with 10 mM glycine-HCl (pH 1.5) for 5 seconds at 5 µL/min.
- Re-equilibration between the sensor surfaces and running buffer is established prior to injection of the next sample.
- Response is monitored as a function of time (sensogram) at 25°C.
- Kinetic parameters (k^a and k^d) are evaluated using the BIAevaluation 4.1 software.
- For the positive control sample, the response units will normally go down with each subsequent run, but the k^d values should be independent of the loss of protein over time. If the k^dvalues are different, then proteins on the chip surface are not usable anymore. Discard the chip and start with the new chip.

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CELLULAR IMMUNOLOGY

I. Preparation of human peripheral blood mononuclear cells (PBMC)

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Equipment

centrifuge (swing out rotor)
Burker chamber
light microscope

Materials and reagents

heparinized tubes (CPT sodium heparin blood collection tubes, BD Vacutainer Systems)
Pasteur pipettes
conical centrifuge tubes
Ficoll-Paque (Pharmacia)
RPMI 1640 with 20 mM HEPES (Gibco)
L-glutamine (Gibco)
penicillin–streptomycin combination (PEST) (Gibco)
fetal bovine serum (FBS) (Gibco)

tissue culture medium (TCM):
RPMI 1640–HEPES
2 mM L-glutamine
penicillin–streptomycin combination (final concentration of 100 units/mL of penicillin G sodium and 100 µg/mL of streptomycin sulfate)
10% heat-inactivated FBS

Turk gentian violet staining solution:
40 mg gentian violet
12.5 mL acetic acid
Make up to 200 mL with distilled water.

Procedure

- Collect venous blood into heparinized 10-mL tubes.
- Save and freeze a small fraction of the plasma in Eppendorf tubes (1 to 2 mL for each tube) for antibody determinations.
- Mix blood in a 1:1 ratio with RPMI 1640 containing 20 mM HEPES solution and carefully layer 7 mL of the resultant suspension on top of 3 mL of Ficoll-Paque in 10-mL conical centrifuge tubes.
- Centrifuge the tubes at 2,800 rpm for 20 min. Collect the interphase with a Pasteur pipette.
- Pool and dilute (≈ 10 times) the cells in RPMI 1640 containing 20 mM HEPES.
- Wash the cells 2 times, first at 1,800 rpm for 10 min and then at 1,200 rpm for 7 min and resuspend the cells in each 10-mL tube in 3 to 5 mL of TCM depending on the density/size of the pellet.
- Pipette 10 µL of the resultant cell suspension in TCM into 40 µL of TURK for counting of the lymphocytes in a Burker-chamber.

Alternative preparation of whole blood cells for cultures

- Collect 10 mL of venous blood as above.
- Pipette 10 μL of cell suspension into 30 μL of Turk for counting of the lymphocytes.
- For the cultures, dilute the blood 2, 5, or 10 times in TCM.

Reference

Smedman L, Joki A, da Silva APJ, Troye-Blomberg M, Aronsson B, Perlmann P. 1994. Immunosuppression after measles vaccination. Acta Paediatr 83(2):164-168.

II. Antigen preparations and peptides for cellular work

II:A. Crude parasite antigen (Percoll-band)

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For working with parasites and Percoll, please refer also to PARASITES and for crude antigen, please refer to SEROLOGY, section III, ELISA.

Equipment

centrifuge (swing out rotor)
sonicator
freezer at −70 °C

Materials and reagents

RPMI 1640–HEPES
PBS
conical centrifuge tubes
Pasteur pipettes
0.45-μm filters (Millipore)

60% Percoll:
60 mL Percoll
7 mL 10× PBS
33 mL 1× PBS

Procedure

- Prepare parasite extracts from late stage cultures. Wash them once in RPMI 1640–HEPES and suspend them to 10% hematocrit in cold RPMI 1640–HEPES.
- Layer 2 mL of extract gently on top of 2.5 mL of cold 60% Percoll in 10-mL conical centrifuge tubes.
- Centrifuge the tubes at 1,500 × *g* for 15 min at 4 °C. Collect the interphase with a Pasteur pipette, pool and wash the cells 3 times with cold PBS (1,500 × *g* for 7 to 8 min), and resuspend them in a small volume of PBS. The interphase contains trophozoites and schizonts, while rings and uninfected erythrocytes are found in the pellet.
- Sonicate (25w) the cells with short intervals for 2 min, determine protein content, and dilute to about 2 mg/mL.
- Centrifuge the sample at 1,500 × *g* for 10 min, filter-sterilize it, make a final protein determination, then store it in aliquots of 1 mg/mL in a −70 °C freezer.

Reference

Troye-Blomberg M, Perlmann H, Patarroyo ME, Perlmann P. 1983. Regulation of the immune response in *Plasmodium falciparum* malaria II. Antigen specific proliferative responses in vitro. Clin Exp Immunol 53(2):345-353.

II:B. Desalting of synthetic peptides

*by **Marita Troye-Blomberg***
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Equipment

speed-vac

Materials and reagents

Sep-Pak C_{18} cartridge (Waters)
5-mL syringe
distilled water
methanol

Procedure

- Use a Sep-Pak C_{18} cartridge connected with a 5-mL syringe to desalt peptides. Prewet the cartridge 3 times with 1.5 mL of methanol and wash it 2 times with 5 mL of water before use.
- Dissolve 10 mg of peptide in 10 mL of distilled water (or 10% methanol in distilled water) and pass the solution 3 times through the cartridge.
- Wash out the salts 2 times with 5 mL of distilled water, elute the peptide 2 times with 1.5 mL of 60% methanol and 2 times with 1.5 mL of 100% methanol.
- Pool the eluates, evaporate them in a speed-vac, weigh them, dissolve them in distilled water at 1 mg/mL, freeze them, and store them frozen.

Reference

Troye-Blomberg M, Riley EM, Perlmann H, Andersson G, Larsson A, Snow RW, Allen SJ, Houghten RA, Olerup O, Greenwood BM, Perlmann P. 1989. T and B cell responses of *Plasmodium falciparum* malaria-immune individuals to synthetic peptides corresponding to sequences in different regions of the *P. falciparum* antigen Pf155/RESA. J Immunol 143(9):3043-3048.

III. T-cell proliferation

*by **Marita Troye-Blomberg***
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Equipment

incubator (37 °C, 5% CO_2 in a humid atmosphere)
scintillation counter
cell harvester (TomTec Cell Harvester)

Materials and reagents

round-bottomed 96-well microplates (Costar 3799, Corning)
(6-^{3}H)-thymidine (specific activity 2 Ci/mmol; Amersham)
TCM (tissue culture medium), see CELLULAR IMMUNOLOGY, section I
peripheral blood mononuclear cells (PBMC)

Procedure

- Dilute PBMC in TCM to 1×10^6 cells/mL and seed 1×10^5 cells/well in 96-well round-bottomed microplates in triplicates per test.
- Add antigens (parasite extracts or recombinant proteins) or peptides at 2 to 10 μg/mL representing epitopes of interest at the initiation of the culture.
- Incubate the plates for 5 days at 37 °C in an atmosphere of 5% CO_2.
- Harvest 100 μL for cytokine determination at the time of pulsing, and then pulse with 1 μCι [6-^{3}H]-thymidine in 100 μL of TCM for 18 h.
- Harvest the cells and measure the thymidine incorporation by liquid scintillation.
- Results are expressed as stimulation index (SI), defined as mean cpm of test triplicates divided by mean of cpm of control triplicates. An SI $\geq$ 2.5 is considered positive.

Reference

Troye-Blomberg M, Riley EM, Perlmann H, Andersson G, Larsson Å, Snow RW, Allen SJ, Houghten RA, Olerup O, Greenwood BM. Perlmann P. 1989. T- and B-cell responses of *Plasmodium falciparum* malaria-immune individuals to synthetic peptides corresponding to sequences in different regions of the *P. falciparum* antigen Pf155/RESA. J Immunol 143(9):3043-3048.

IV. Interleukin ELISPOT

by ***Gehad El Ghazali***
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The following description should be considered as a guideline on how to stimulate and analyse cells for IFN-γ production by the ELISPOT technique. Conditions for cell preparation, stimulation, and the ELISPOT may be changed to fit with the test situation.

Equipment

incubator (37 °C, 5% CO_2 in a humid atmosphere)
multi-channel pipette
dissection microscope (40×)

Materials and reagents

nitrocellulose plates (Millipore Corp.)
mAb anti-human IFN-γ, 7-B6-1-Biotin (Mabtech)
biotinylated mAb anti-human IFN-γ, 7-B6-1-Biotin (Mabtech)
streptavidin–alkaline phosphatase (Mabtech)
BCIP/NBT substrate (Bio-Rad Laboratories)
TCM (tissue culture medium), see CELLULAR IMMUNOLOGY, section I
5-mL round-bottomed tissue culture tubes (Falcon 2058, BD Labware)
PHA (phytohemagglutinin)
PBS (filter-sterilized phosphate buffer saline) (pH 7.2)
FCS (fetal calf serum)

Procedure

Stimulation of cells can be carried out directly in the ELISPOT plate, but better results may be obtained if cells are first stimulated for a shorter period in culture tubes. In the following description cells are incubated for 2 to 4 h in tubes and thereafter transferred to ELISPOT plates for further incubation.

- Prepare sterile PBMCs.
- Count cells and suspend them in TCM to a concentration of 2×10^6 cells/mL.
- Transfer 0.5 to 1 mL of cell suspension to 5-mL round-bottomed tissue culture tubes.
- Add antigens (parasite extracts or recombinant proteins) or peptides representing epitopes of interest at a concentration of 2 μg/mL at the initiation of the culture. As a positive control, add phytohemagglutinin (PHA) at a concentration of 5 μg/mL.
- Incubate cells at 37 °C for 2 to 4 h.

Coating of ELISPOT plates

- Dilute the coating antibody (mAb anti-human IFN-γ, 7-B6-1-Biotin) to 15 μg/mL in filter-sterilized phosphate buffer saline (PBS).
- Add 100 μL/well to the nitrocellulose plates and incubate them overnight at 4 °C.

Transfer of cells to the nitrocellulose plate (sterile)

Before transferring stimulated cells to the coated ELISPOT plates, wash them 4 times with sterile PBS (200 μL) to remove unbound coating antibody.

- Add stimulated cells to the plates. For IFN-γ, a cell concentration of 20,000 to 50,000 cells/well is usually suitable. If the number of cells exceeds 250,000 cells/well, this may result in blurry spots due to multiple cell layers (especially in the wells with cells stimulated with PHA).
- Incubate cells for 10 to 40 h at 37 ^{o}C in a humid atmosphere.

Addition of the secondary antibody (nonsterile)

- Remove cells by washing them 6 times (200 µL/well) using PBS (multi-channel pipette).
- Dilute biotinylated mAb (anti-human IFN-γ 7-B6-1-Biotin) to 1 µg/mL in PBS with 0.5% fetal calf serum (PBS-0.5% FCS) and add 100 µL/well.
- Incubate the plates for 2 to 4 h at room temperature.
- Wash them in PBS 6 times (200 µL/well).
- Add 100 µL of streptavidin–alkaline phosphatase (diluted 1:1000 in filtered PBS-0.5% FCS) and incubate the plates for 1 to 2 h at room temperature.
- After washing them in PBS 6 times (200 µL/well), add 100 µL of substrate and incubate them until spots emerge (may be up to 1 h).
- Stop color development by washing the plates under running tap water (3 times at 200 µL/well).
- Leave the plate to dry; inspect and count the spots with a dissection microscope (40×).

One-Step ELISpot kits:
Very sensitive and easy to use kits are available at Mabtech AB.

Reference

Elghazali G, Esposito F, Troye-Blomberg M. 1995. Comparison of the number of IL-4 and IFN-γ secreting cells in response to the malaria vaccine candidate antigen Pf155/RESA in two groups of naturally primed individuals living in a malaria endemic area in Burkina Faso. Scand J Immunol 42(1):39-45.

V. Determination of experimental cerebral malaria and serum harvested from mice after *Plasmodium berghei* infection via multiplex bead array analysis

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Materials and Reagents:

- 70 µm cell strainers
- 20 ml syringes
- PBS
- Sample Buffer: 0.05% Tween 20 and protease inhibitor cocktail in PBS
- Table centrifuge (capable of 15000g)
- Anaesthetics (e.g. for mice, 25mg/ml Ketamine, 2.5mg/ml Xylazine, 100µl/25g animal by intraperitoneal injection)
- Mouse surgical equipment tools (scissors, tweezers, etc)
- 15ml conical, polypropylene tubes, (e.g. BD Falcon)
- 12 x 75mm sample acquisition tubes (e.g. BD Falcon)
- Multiplex Bead Array kit (e.g. BD Falcon, eBioscience)
- Flow Cytometer (capable of detecting two distinct emissions, according to kit requirements)

Organ harvest

- Anaesthetize mouse and dissect to expose heart and organs of interest
- If serum analysis is required, draw blood from the hepatic artery
- Perfuse the animal using cardial perfusion with ~20 ml PBS
- Isolate whole or pieces of organ required for analysis in sample buffer and determine weight of each
- Homogenize organs in 2 ml sample buffer by squishing organ through 70µm cell strainer, transfer cell suspension to 2 ml reaction tubes
- Centrifuge (10 min, 15000 g) and retain supernatant (Store at -20°C)
- Centrifuge supernatant prior to analysis
- Repeat centrifugation step each time a sample is thawed for new analysis

Cytokine analysis

To determine cytokine concentrations in organs and serum use a multiplex bead array kit according to manufacturer's protocol.
The cytokines are "captured" by beads of known size and fluorescence conjugated with a specific antibody and subsequently quantified by fluorescence readout obtained by adding a detection antibody.
Recombinant cytokine standards are provided in the kit and a dilution series prepared to produce a standard curve against which experimental samples are measured to determine the unknown sample cytokine concentration. Computational analysis of data readout should be conducted with suitable software, such as the FCAP Array Analysis by Softflow Inc.

Problem solving

- If cytokine concentration readout is undetectable, increasing the mass of organ in the homogenate may increase the concentration into the “detectable” threshold.

- The “detectable” cytokine threshold can be optimized by adjusting standard concentrations to those in the range expected in the experimental readout

- Mouse group numbers should be large (for example 10 mice) in order to obtain statistically significant data readouts. The cytokine concentrations between individual mice vary substantially.

Reference

Mohamed F. Elshal, J. Philip McCoy, Multiplex Bead Array Assays: Performance Evaluation and Comparison of Sensitivity to ELISA. 2006 Methods. 2006 April; 38(4): 317–323.

VI. Determination of experimental cerebral malaria (ECM)

*by **Matthew Lewis, Jessica Kehrer***
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Materials and Reagents:

- C57BL/6 mice
- *Plasmodium berghei* strain ANKA
- Evans Blue (T-1824) (1 mg/ml in PBS)
- Anaesthetics (e.g. for mice, 25mg/ml Ketamine, 2.5mg/ml Xylazine, 100µl/25g animal by intraperitoneal injection) Mouse surgical equipment tools (scissors, tweezers, etc)

The standardized model for experimental cerebral malaria (ECM) is the *Plasmodium berghei* ANKA parasite and the C57BL/6 mouse strain. Between day 5 and day 12 post-infection susceptible mice present with symptoms of experimental cerebral malaria which rapidly progresses to coma and death. Onset is delayed depending upon the route of infection, i.e. injection of sporozoites and subsequent liver stage development will take a further three to four days compared to those infected with parasitized erythrocytes, until mice present with clinical symptoms.

Typical ECM symptoms:

Lethargy
Ataxia
Lacrimation: “milky eyes”
Seizures
Coma

Symptoms progress rapidly and animals will usually die within 24h. During ataxia, mice become largely unresponsive to most external stimuli but will move if touched or manipulated. The evolution of ECM symptoms can be quantitatively analyzed by assessing mice according to the "Rapid Murine Coma and Behavior Scale", described by Carroll *et al* 2010. During coma, mice become unresponsive to most stimuli, both external and physical, but will still exhibit the tail pinch reflex. To test for coma, lift the mouse and gently let it drop from several centimetres. Ataxic animals will extend their front paws to cushion their landing, whereas comatose animals will not.

One hallmark symptom of ECM is permeabilization of the brain microvasculature. This can be determined and quantified by intravenous injection of Evans Blue prior to sacrificing the animal:

- Inject 100-200µl Evans Blue solution intravenously. Dye distribution will become visible almost immediately.
- Sacrifice mice after 1h and perfuse by cardial perfusion of ~20ml PBS
- Isolate brain and assess vascular leakage: affected brains will become visibly blue
- For quantification of vascular permeabilization, incubate in 3 ml formamide for 48h at 37°C
- Measure absorbance of solution with a spectrometer at 620nm

References

Carroll, R. W., M. S. Wainwright, et al, 2010. "A rapid murine coma and behavior scale for quantitative assessment of murine cerebral malaria." PloS one 5(10).

Hawkins BT, Egleton RD. 2006. Fluorescence imaging of blood-brain barrier disruption. J Neurosci Methods. 15;151(2):262-7.

Schmidt, K. E., B. Schumak, S. Specht, B. Dubben, A. Limmer, and A. Hoerauf. 2011. Induction of pro-inflammatory mediators in *Plasmodium berghei* infected BALB/c mice breaks blood-brain-barrier and leads to cerebral malaria in an IL-12 dependent manner. Microbes Infect 13:828-836.

VII. Induction of Experimental Cerebral Malaria susceptibility by transfer of mature lymphocyte populations.

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We developed a novel cell transfer protocol to test individual cell population ability to induce susceptibility to Experimental Cerebral Malaria (ECM). This protocol is based on pre-sensitizing donor mice with irradiated blood stage parasites before cell transfer. The protocol has been tested to induce ECM susceptibility to genetically resistant mouse strains using purified $CD8^+$ T cell populations.
This protocol provides the means to activate immune cells by infected red blood cells (iRBC) in the absence of disease development.

Equipment
Centrifuge (15ml tube)
Irradiator (for 20Krad)
Facs Cell Analyzer
FacsAria Multicolour Cell sorter

Materials and reagents
PBS 1X
PBS 1X, FCS 2%
FACS buffer (PBS1X, 2%FCS, 2mM NaN_3)
Heparin (5000 UI/ml)
Eppendorf tubes
50ml + 15ml Falcon tubes
Surgical tools
Small petri dishes
Sterile gauze
Plasmodium berghei ANKA- GFP infected red blood cells (iRBC)

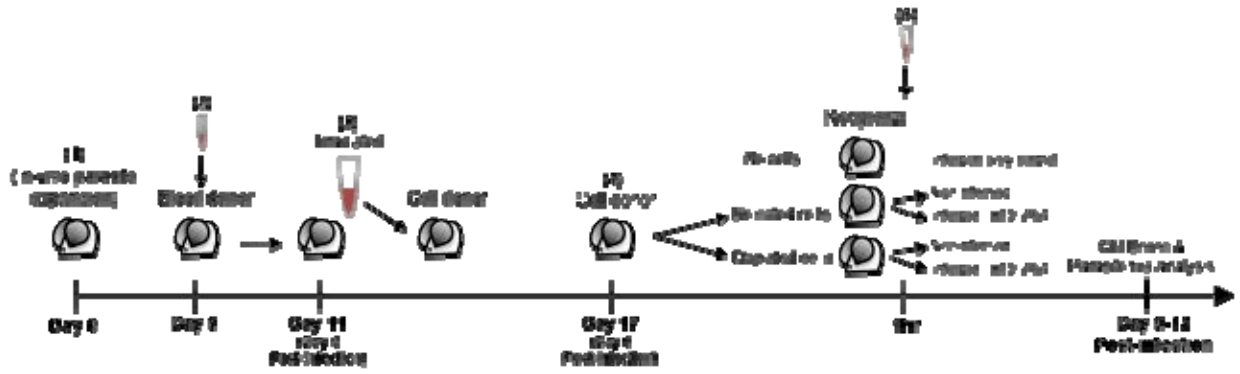

Figure 1. Timeline and schematic representation of the protocol

Procedure

(1)Infect initial mouse for parasite expansion* $1x10^6$ iRBC/100μl in PBS1X intraperitoneally (i.p) from frozen vial of P.berghei ANKA-GFP. Allow expansion of parasite and measure daily parasitemia percentage until mouse reaches between 1-5% iRBCs.

- To measure parasitemia percentage, take a drop of tail blood from infected mouse into 600μl FACS buffer. Using a Facs cell analyzer, measure parasite GFP fluorescence as indicator of iRBC percentage. Using this percentage of iRBC, calculate the volume of blood to collect from infected mouse to then infect blood donor mouse $1x10^6$ iRBC/100μl, in PBS1X.

(2)To collect blood, bleed infected mouse via mandibular vein puncture into an eppendorf containing 10μl Heparin, pipette up and down to prevent clotting. Take from this the previously calculated volume for iRBC and resuspend in PBS1X. Inject iRBCs i.p into blood donor mouse. Allow parasite expansion for 6 days.

(3)On day 6 post-infection (day 11)/or upon display of ECM symptoms in blood donor mouse, measure parasitemia (as described). Calculate from parasitemia percentage the volume of blood to collect that will be irradiated and injected $4x10^6$ iRBC/100μl in PBS1X, per cell donor mouse.

- For irradiation, collect blood from blood donor mouse in an eppendorf (as described, with 10μl heparin, pipette well) and place immediately the eppendorf in a 50ml falcon tube containing ice (do not dilute blood in PBX1X in this step).
- Place 50ml falcon tube containing ice and eppendorf with collected blood in Irradiator and irradiate at 20,000rad. (Obs: Irradiation of parasite to prevent recipient development of ECM and parasitemia).
- After irradiation, take volume of blood and dilute in PBS1X (final concentration, $4x10^6$ iRBC/100μl per mouse) and inject i.p into cell donor mice.

(4)On day 6 post-injection (day 17) sacrifice cell donor mice and remove spleens.

- Place spleens in a petri dish on ice containing PBS1X, FCS2%.
- In a new petri dish smash each spleen in 2ml of PBS1X, FCS2% in-between sterile gauze using the tips of two sterile surgical forceps. Pipette cell suspension into a 15ml Falcon on ice, and wash petri dish with additional 2ml PBX1x, FCS2%. (2 spleens per falcon can be used).
- Proceed with a standard FACS staining protocol for the desired cell population and sorting for purified populations.
- After sorting, collect cells and wash in sterile PBS1X. Count cells and resuspend in PBS1X to a final concentration of $3x10^6$ cells/100μl, per mouse. Inject cells intravenously into recipient mice groups.

(5)Infect i.p appropriate recipient mice groups 1 hour after cell transfers with $1x10^6$ iRBC/100μl of P.berghei ANKA-GFP.

- Monitor Recipient mice for parasitemia and ECM development from day 5 PI.

* All mice used throughout the protocol need to be carrying the same H-2 haplotype.

VIII. Phagocytosis assays

VIII:A. THP1 phagocytosis by flow cytometry

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Equipment
Flow cytometer
Materials and reagents

FACS tubes	FITC (Sigma)
U-bottom plates	Dihydroethidium (HE: Polybiosciences)
THP-1 cells	NaHCO3, pH 9.0
Enriched parasite suspension	PBS
Ammonium Chloride lysing solution (prepared or commercial)	Foetal calf serum (FCS)
	THP1 medium

Procedure
Erythrocyte opsonization and THP1 phagocytosis

- Enrich parasite culture by magnetic cell sorting (MACS), percoll or plasmion (plasmagel) if late stages required. For early stages, allow enrich parasites to reinvade.
- Suspend parasites to 10^8 cells/mL in fresh 0.5 M sodium bicarbonate, pH 9.0.
- Then label total red cell population with 50 mg/mL of FITC for 30 min at room temperature (20-25^{o}C) with constant agitation.
- Wash 3 times in cold PBS.
- Then label infected RBCs with HE (5 µg/mL) for 30 min at room temperature.
- Distribute 100 µl to U-bottom plate containing antibody preparation (at a ratio of 20 RBCS to 1 phagocyte).
- Opsonise for 30 min at room temperature in the dark with gentle agitation or resuspend every 10 min if static conditions.
- Meanwhile Count THP1 cells using trypan blue to exclude dead cells.
- Adjust to 5x 10^4 THP1 cells /mL using THP1 medium (RPMI-GlutaMax /2% FCS/ penicillin- streptomycin and L-glutamine).
- Transfer 1 mL to each FACS tube and incubate at 37^{o}C, 5%CO_2 for 1 h.
- Meanwhile wash opsonised RBCs 3 times with RPMI/1% FCS at room temperature.
- Resuspend opsonised cells with 100 µl medium.
- Transfer opsonised cells to FACS tubes containing THP1 cells (at a ratio of 20 targets: 1 phagocyte). Have tube(s) with only THP-1 cells.
- Incubate for 30 min at 37^{o}C with gentle agitation.
- To stop phagocytosis, add 1 mL of ice cold PBS/1% FCS or centrifuge at 4 degrees at 300xg for 3 min, remove supernatant
- Lyse uningested RBCs with 1 mL of lysing solution indicated below or use BD FACS lysing solution according to manufacturer's instructions.
- Wash twice with 3 mL ice cold PBS/1% FCS.
- Resuspend in 1 ml cold PBS/1% FCS or fixing solution (e.g. 1- 2% cold paraformaldehyde in PBS).

- During FACS acquisition, keep on ice and in the dark.
- Identify and gate viable THP1 cells via light scatter characteristics using tube with THP1 cells only and negative control (no antibody).
- Distinguish phagocytised non-infected or infected RBCs using FITC (FL1) and HE (FL2 or FL3) fluorescence, respectively.
- Acquire up to 30,000 cells.

Ammonium Chloride lysing solution 10X
89.9g NH4CL
10g KHCO3
370mg Na_4EDTA
Store for no longer than 6 months at 2-8 degrees tightly closed. Use at 1X. Prepare fresh each time (daily) and store at room temperature. Discard any unused portion.

Lysing procedure

- Add 1 mL of lysis solution to each tube.
- Immediately vortex for 30 sec.
- Incubate at RT for 3 min or until clear. Do not exceed 3 min.
- Centrifuge at 200g/5 min/room temperature 20-25°C
- Suction supernatant while observing cell pellet.
- Wash 2 times with 3 mL ice cold PBS/1% FCS.
- Resuspend in 1 ml PBS/1%FCS buffer or fixing solution (e.g. 1- 2% cold paraformaldehyde) for FACs analysis.

NOTES

- THP-1 cells grow better in RPMI-GlutaMax (Gibco-Invitrogen), supplemented with 5% FCS.
- Use cells when in log phase of growth. Dead cells should not exceed 5%.
- Phagocytosis is rather rapid (already seen within 5 min), so the timing has to be precise to avoid digestion of ingested cells.
- Phagocytosis can equally be carried out in U-bottom plates under static conditions.
- Instead of 37°C incubator, a water bath can be used for phagocytosis.
- For rosette-forming parasites, use heparin to disrupt rosettes.

References

Awah N, Balogun H., Achidi E., Mariuba L.A., Nogueira P.A., Orlandi P, Troye-Blomberg, M, Gysin, J, Berzins K.. 2011. Antibodies to the *Plasmodium falciparum* rhoptry protein RAP-2/RSP-2 in relation to anaemia in Cameroonian children. Parasite Immunol, 33(2) :104–115.

Ataide R, Hasang W, Wilson DW, Beeson JG, Mwapasa V, et al. 2010. Using an improved phagocytosis assay to evaluate the effect of HIV on specific antibodies to pregnancy-associated malaria. PLoS One 5: e10807.

Ghumra A, Khunrae P, Ataide R, Raza A, Rogerson SJ, Higgins MK, Alexandra Rowe J. 2011. Immunisation with recombinant PfEMP1 domains elicits functional rosette-Inhibiting and phagocytosis-Inducing antibodies to *Plasmodium falciparum*. PLoS ONE 6(1): e16414.

Tippett E, Fernandes LA, Rogerson SJ. Jaworowski A. 2007. A novel flow cytometric phagocytosis assay of malaria-infected erythrocytes. J Immunol Methods 325: 42–50.

VIII:B. *In vitro* phagocytosis assay by microscopy

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Equipment-Light

Microscope MgX100
Vortex
37 degree Water bath
Eppendof tubes
CO_2 humidified Incubator
Staining rack and troughs
Blow dryer (Hair dryer)

Materials and reagents

Giemsa
May-Grünwald Giemsa
CultureSlideChambers, (LabTek, BD Falcon, Franklin Lakes, NJ, USA)
Phagocytosis medium/monocyte medium
Peripheral blood mononuclear cells (PBMCs) isolated from freshly collected buffy coats or whole blood
Enriched parasite suspension
Monocyte/Phagocytosis medium
RPMI 1640 with 2 mM glutamine,
10 mM Hepes, 2 g/l sodium bicarbonate,
10 mM sodium pyruvate,
2×10^{-5} M β_2-mercaptoethanol
10 mg/l gentamicin
20% foetal calf serum (decomplement at 56 °C for 30 min)

Procedure

PBMC s

- Isolate PBMCs from freshly collected whole blood or buffy coat.
- Re-suspend PBMCs in monocyte medium at a density of 2.5×10^7 cells/mL.
- Add 1ml to wells of LabTek slide chambers.
- Incubate at 37^{o}C humid incubator.
- Adhere for 2 h to isolate monocytes.
- Wash off non-adherent cells by repeated washing with warm monocyte medium.

Opsonisation

- Use an enriched parasite culture or a culture of ~10% parasitemia at the minimum. The stage to use will depend on the specificity of your antibodies.
- Wash cultures with phagocytes medium 3 times.
- Add 25-100 µl of various antibody preparations to be used/controls into labeled Eppendof tubes.
- Add 2.5×10^6 cells into each tube-vortex well.
- Incubate for 30 min at 37^{o}C (H_2O bath).
- Wash 3 times with 500 µl WARM phagocyte medium (500xg for 2 min).
- Resuspend pellet with 200 µl of warm phagocyte medium.

Phagocytosis

- Transfer suspension to labtek with adherent monocytes.
- Incubate for 1h at 37^{o}C in a 5% CO_2 incubator.
- Remove plastic chamber of Labtek.
- Wash slides with warm PBS (37^{o}C) in a trough.
- Quickly dry with a hair dryer.
- Stain with May-Grünwald Giemsa.

Staining procedure

- Flood slide with 0.5 mL of May-Grünwald Giemsa (undiluted) for 1 min (The methanol in the stain fixes the cells).
- Dilute May–Grünwald Giemsa on the slide by adding 0.5 mL dH2O to the slide for 1 min.
- Pour off stain-do not rinse.
- Add Giemsa stain (diluted 1:10) for 5 min.
- Rinse slide with dH2O.
- Quickly blow dry.
- Microscopy-oil immersion.
- Determine the phagocytic index. To determine the phagocytic index, count infected or non-infected RBCs ingested per 500 phagocytes. Thus, the phagocytic index is the total number of internalized RBCs per total number of macrophages counted expressed as a percentage.

Phagocytic index (AI)

$$\frac{\text{RBC ingested}}{\text{No. of phagocytes}} \times 100$$

NOTES

- May–Grünwald–Giemsa gives a better visualisation of the parasite within the monocyte, which is different from the densely stained nuclei of the monocyte.
- Isolate PBMCs from freshly collected blood or buffy coats.
- Work rather fast, ensure monocytes always at 37^{o}C. Use a 37 degree water bath to keep the medium warm throughout.
- The fluorescence of attached but non-internalised cells can be quenched using 0.4% trypan blue (w/v).

References

Groux H, Gysin J. 1990. Opsonization as an effector mechanism in human protection against asexual blood stages of *Plasmodium falciparum*: functional role of IgG subclasses. Res. Immunol. 141: 529–542.

Awah NA, Troye-Blomberg M, Berzins K, Gysin J. 2009. Mechanisms of malarial anaemia: Potential involvement of the *Plasmodium falciparum* low molecular weight rhoptry-associated proteins Acta Tropica, 112:295-302.

Kumaratilake ML and Ferrante A. 2000. Opsonization and phagocytosis of *Plasmodium falciparum* merozoites measured by flow cytometry. Clin. Diagn. Lab. Immunol, 7:9-13.

VIII:C. Assessment of the uptake of a recombinant BCG expressing the *P. falciparum* circumsporozoite by bone marrow-derived macrophages *in vitro*

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Materials and reagents

Middlebrook /H11 agar plates ().
Lysis buffer (0.2% Triton X-100 in sterile 1X PBS).
24-well culture plate
Incubator
Complete DMEM (DMEM 500 ml, Pyruvate Sodium 5 ml, Mercapto ethanol (2-ME) 500 µl, Glutamine 5 ml, Hepes 10 ml, Penicilin (100UI/ml) Steptomycin (100mg/ml) 5 ml, Fetal Calf Serum 50 ml).
ACK RBC-lysis buffer (0.15 M NH4Cl, 10 mM $KHCO_3$, 0.1 mM Na_2-EDTA in dH_2O and adjusted pH to 7.2–7.4 with 1 M HCl
L929 cell-line conditioned medium.

Experimental steps

1 Generation of Bone marrow derived macrophage (BMM) from mice marrow cells

¤ Caution! Work aseptically in laminar flow hood!

- Remove aseptically femurs and tibias and gently take away adherent tissue.
- Cut the ends of the bone and flush out the marrow with plain RPMI 1640 using 23 G needle into a sterile petri dish.
- Prepare the single cell suspension by passing the marrow through the 23 G needle with syringe to disperse to single cells.
- Remove the RPMI by centrifugation and lyse the red-blood cells by re-suspending the cells in ACK buffer (5 minutes) and wash the cells twice and re-suspend in complete medium.
- Count and seed the cells at a concentration of 5 x 10^5- 10^6 cells / well in 1ML in Complete DMEM supplemented with 20 % L929 conditioned medium as the source of macrophage colony-stimulating factor (M-CSF).
- Change the medium every second day until the culture reaches 80-90% confluence.
- Replace medium with complete RPMI or DMEM WITHOUT PEST (day 6 or 7) and keep the cells at 37 ° C, 5% CO_2 for 24 hours before infecting with rBCG.

2 Infecting BMM with rBCG

- Prepare your cells (macrophages dendritic cells) in complete RPMI or DMEM and culture them in a 24-well plate according to the cell culture protocol to achieve appropriate confluence[1].
- Dilute rBCG in complete RPMI or DMEM without antibiotics and infect macrophages or dendritic cells with multiplicity of infection (MOI) 10:1 and incubate for 4 hours at 37˚C.
- Wash the cell monolayer (very gently) to remove unabsorbed rBCG three times with pre-warmed RPMI or DMEM (serum and antibiotic free). Vigorous washings could destroy cell monolayer especially cells that are loosely adhered.

- Seed the last wash solution in Middlebrook /H11 agar plates and incubate the plates at 39-40˚C to check extracellular rBCG growth by counting colony-forming units (CFUs).
- Treat the cell monolayer with gentamicin (100μg/ml) in complete RPMI or DMEM for 1 hour at 37˚C to kill the remaining extracellular bacteria[2]. This should be the one to be cultured to see the background bacterial growth.
- Wash (very gently) three times with prewarmed RPMI or DMEM (serum and antibiotic free) to remove all gentamicin from the cultures[3].
- To measure phagocytosis (0 hour), lyse cells with lysis buffer (500 µl /well) for 15 min at 37˚C to release bacteria from the cells and plate cell lysates on Middlebrook /H11 agar plates at appropriate serial dilutions (10, 10^2, 10^3, 10^4 etc…).
- Incubate the plates at 39-40^0C for 2-3 weeks.
- Determine the number of rBCG by counting the CFUs on the Middlebrook /H11 agar plates.

NOTES:

This method can be applied to measure dendritic cell phagocytic activity.

[1]Cell confluence might vary from cell type to cell type during culturing. It is recommended to establish an appropriate confluence, which never exceeds 90-95% until you finish the experiments.

[2]A complete removal of extracellular bacteria is very important.

rBCG is very “sticky” and bacteria remaining outside of the cells can grow very well in complete RPMI or DMEM and therefore might interfere during evaluation.

[3] Removal of the antibiotics is very important, otherwise they can be internalised and this will affect bacterial growth inside cells.

VIII:D. Opsonic monocyte phagocytosis assay

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Equipment

Vario-MACS magnet (MiltenyiBiotec)
MACS CS-column (MiltenyiBiotec)
3-way stopcock (MiltenyiBiotec)
10-mL syringe
5-mL syringe
0.8-mm needle
0.6-mm needle
50-mL Falcon tubes
Counting chamber
Microscope
96-well plates (rounded bottom)
centrifuge (tube and plate rotor)
CO_2 incubator

Materials and reagents

RPMI 1640
RPMI 1640/HEPES/sodium bicarbonate/ gentamycin solution (MCM; see PARASITES, section I:A)
PBS supplemented with 2% heat inactivated FBS
Aphidicolin(see PARASITES, section I:F)
Ammonium chloride lysing solution (15mM NH_4Cl, 10 mM $NaHCO_3$, 1 mM EDTA)
THP-1 cell culture media (RPMI 1640, 2 mM HEPES, 20 mM glutamine, 0.85 g/L $NaHCO_3$, 10% heat inactivated FBS, 1000U/ml Penicillin, 1mg/ml Streptomycin)
Ethidium bromide 10mg/ml

Procedure

- Before starting the assay block for at leastone hour two 96-well plates (one for the opsonization and a separate one for the phagocytosis) using150 μl of PBS 2% FBS per well
- Isolate mature trophozoite-infected erythrocytes using VarioMACS magnet (see PARASITES, section IV:J, use RPMI instead of PBS/BSA). Determine the final parasitemia and the total cell number
- Wash once with MCM and re-suspend in a 250 μg/mlethidium bromide solution (1:4000, from stock 10 mg/ml)prepared in MCM, add the necessary volume to obtain a concentration of $3.3X10^7$pRBCs/ml
- Remove the blocking solution from one of the 96-well plates (opsonization plate) and add 30 μl of the pRBCssuspension in each well
- Incubate for 10 minutes at room temperature protected from light
- Wash three times in MCM (200 μl per well, 300g, 2 minutes)
- Re-suspend in 30 μl of antibody/serum solutionwith the desired concentrations. Always include a positive control (e.g. rabbit anti human erythrocyte antibody

ab34858 90 mg/ml ABCAM, 1:100 dilution) and a control without any antibody (unopsonized control)
- Incubate during 45 minutes in dark at 37°C
- In the meantime begin to prepare the THP-1 cells. Remove the cells form the culture flask, spin them down, remove the supernatant, re-suspend the pellet in THP-1 cell culture medium and spin again, remove the supernatant and re-suspend in 1 ml of THP-1 cell culture medium (spin at 500g for 5 minutes)
- Determine the number of cells per ml in the solution above and add more medium to obtain a final concentration of $5X10^5$ cell/ml
- Remove the blocking solution from the other 96-well plate (phagocytosis plate) and add 100 μl of the THP-1 cell suspension in each well
- Once the antibody/serum incubation time has finished, wash the pRBCs three times with MCM and finally re-suspend in 100 μl of THP-1 cell culture medium (spin at 300g, 2 minutes)
- Transfer 50 μl of the opsonized-pRBCs suspension to each well in the phagocytosis plate (since there are 100 ul of pRBCs per each antibody/serum dilution, there is enough sample to run duplicates in the phagocytosis plate)
- Incubate for 40 minutes at 37°C, 5% CO_2
- Stop the phagocytosis by centrifugation at 4°C (500g, 5 minutes)
- Replace the 150 μl supernatant with room temperature ammonium chloride lysing solution and mix by pipetting, incubate for 3 minutes (this will lyse the uningested RBCs)
- Stop the lysis adding 100 μl of ice cold PBS 2% FBS, and wash three times
- After the final wash, re-suspend in 200 μl of PBS 2% FBS and analyze by flow cytometry. Gate for THP-1 cells and determine the percentage of ethidium bromide positive cells

Comments
- THP-1 cell culture should be started at $2.5X10^5$ cells/ml and subcultured when cell concentration reaches $8X10^5$ cells/ml. Culture should never exceed $1X10^6$ cells/ml.
- The average doubling time of the THP-1 cell line is very variable (30-50 hours), therefore before starting experiments is recommended to estimate the doubling time of the particular batch of cells
- For the phagocytosis experiments set a flask two days before the experiment starting with $1\text{-}2X10^5$ cells/ml. Keep in mind the doubling time of the cell batch used and the final amount of cells needed for the experiment
- Parasites should be synchronized using sorbitol (see PARASITES, section IV:B) at least twice consecutively and the parasitemia should be as high as possible (10%)
- Aphidicolin can be used to arrest parasite growth and obtain a more homogenous population for the assay(see PARASITES, section I:F)
- After the isolation of the pRBCs with the MACS magnet use ONLY samples with final parasitemia of at least 80%
- Don't allow the phagocytosis to procede for more than 40 minutes
- It is important to keep all media preheated to 37 °C and to minimize handling time outside the 37 °C incubator, particularly during the phagocytosis step

References

Barfod, L., Dalgaard, M. B., Pleman, S. T., Ofori, M. F., Pleass, R. J., & Hviid, L. (2011). Evasion of immunity to *Plasmodium falciparum* malaria by IgM masking of protective IgG epitopes in infected erythrocyte surface-exposed PfEMP1. *Proceedings of the National Academy of Sciences of the United States of America*, *108*(30), 12485–12490. doi:10.1073/pnas.1103708108

Barfod, L., Dobrilovic, T., Magistrado, P., Khunrae, P., Viwami, F., Bruun, J., Dahlbäck, M., et al. (2010). Chondroitin sulfate A-adhering *Plasmodium falciparum*-infected erythrocytes express functionally important antibody epitopes shared by multiple variants. *Journal of immunology (Baltimore, Md.□: 1950)*, *185*(12), 7553–61. doi:10.4049/jimmunol.1002390

Ghumra, A., Khunrae, P., Ataide, R., Raza, A., Rogerson, S. J., Higgins, M. K., & Rowe, J. A. (2011). Immunisation with recombinant PfEMP1 domains elicits functional rosette-inhibiting and phagocytosis-inducing antibodies to *Plasmodium falciparum*. *PloS one*, *6*(1), e16414.

Tippett, E., Fernandes, L. a, Rogerson, S. J., & Jaworowski, A. (2007). A novel flow cytometric phagocytosis assay of malaria-infected erythrocytes. *Journal of immunological methods*, *325*(1-2), 42–50.

IX. Detection of cytokine-gene polymorphism

IX:A. Extraction of human DNA from peripheral blood or buffy coat

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Equipment

incubator
centrifuge
gel electrophoresis apparatus
speed-vac
gel drier or oven
spectrophotometer
camera
automated thermal cycler
microscope with UV light

Materials and reagents

lysing buffer:

1.6 M sucrose
50 mM Tris–HCl (pH 7.5)
25 mM $MgCl_2$
5% Triton X-100

DNA buffer:

10 mL 1 M Tris–HCl (pH 7.5)
2.5 mL 0.5 M NaCl
2.5 mL 0.5 M EDTA
1.25 mL 20% SDS
Adjust volume to 50 mL using double distilled H_2O.

sterile vacutainers with EDTA (ethylamine tetra-acetic acid)
Falcon tubes, 50-mL
proteinase K solution (Invitrogen 25530 049)
20% SDS
sterile double-distilled H_2O
Pasteur pipettes
Eppendorf tubes
phenol
chloroform
3 M sodium acetate
absolute ethanol
70% ethanol

Procedure

- Draw 1 to 8 mL of venous blood into sterile vacutainers containing EDTA and keep it at room temperature (or frozen).
- Transfer blood into a 50-mL Falcon tube and add 10 to 40 mL of lysing buffer.
- Turn the tube gently upside down for 2 to 3 min.

- Centrifuge the tube at 2,000 rpm for 10 min (with brake off) and discard the supernatant.
- Add 40 µL of proteinase K solution, 40 µL of 20% SDS, 180 µL of DNA buffer, and 340 µL of sterile double-distilled H_2O.
- Mix the sample well by gently pipetting it up and down until it becomes gelatine-like.
- Digest the blood by incubating it overnight at 37 to 42 ^oC.
- Transfer the sample into Eppendorf tube and add 600 µL of phenol.
- Extract the DNA by revolving/mixing the tubes until the solution becomes milky.
- Centrifuge the tube at 13,000 rpm for 10 min at room temperature.
- Transfer the upper aqueous phase (DNA) into a new Eppendorf tube.
- Add 800 µL of chloroform.
- Extract the DNA and centrifuge the tube at 6,000 rpm for 10 min.
- Transfer the upper phase into a new Eppendorf tube.
- Precipitate the DNA by adding one-tenth volume of 3 M sodium acetate and 2.5 volumes of absolute ethanol; allow the tube to sit for 2 hours or overnight at −20 °C. The DNA can be stored this way as well.
- Centrifuge the precipitate for 30 min at 4 °C, wash it gently once with 70% ethanol, dry it in a speed-vac, and gently resuspend the pellet in 25 to 100 µL of distilled water, depending on its size.
- Determine the DNA concentration at OD_{260}; 2 mL of culture may yield ~2 µg of genomic DNA. Visualize ~0.5 µg on a 0.8% agarose gel to see that the DNA runs as a high-molecular weight, somewhat broad band and is thus unsheared and free of RNA.

IX:B. PCR for amplification of nucleotide repeat (VNTR) polymorphisms of cytokine genes

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Equipment

automated thermal cycler
UV-transilluminator
camera

Materials and reagents

dNTPs
primers
Taq polymerase
PCR buffer with $MgCl_2$
sterile H_2O
Taq gold (Applied Biosystems)
agarose gel
0.1% ethidium bromide
CAUTION: This chemical is toxic and mutagenic. Wear protective clothing and use only in a chemical fume hood.

TE buffer (pH 8.0):
10 mM Tris Cl (pH 8.0)
1 mM EDTA (pH 8.0)

Procedure

- Prepare the mix at the following final concentration (total volume 25 μL):
  100-250 ng DNA
  200 nM dNTPs
  0.1 μM of each primer
  1.5 U *Taq* polymerase
  PCR buffer (with $MgCl_2$), 8% (v/v)
  sterile H_2O, 46.4% (v/v)
- Program the automated thermal cycler according to manufacturers' instructions. Use the following guidelines to set annealing temperature and extension time according to primer and product considerations.
- Hot start at 95 ^{o}C for 10 min when using *Taq* Gold.
- Denature the DNA for 1 min at 94 ^{o}C. If the GC content is ≤50%, anneal it at 55 ^{o}C; if it's >50%, anneal at 65 ^{o}C. Extend for 5 min at 72 ^{o}C. Run the program for 30 cycles.
- Prepare 2% agarose gel in TE buffer. Run the gel at 80 to 100 V and stain it with 0.1% ethidium bromide. Visualize the gel under UV light. Take a photograph.

IX:C. PCR for amplification of single nucleotide polymorphisms or RFLP of cytokine genes

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Procedure

- Use the same procedure as for VNTR polymorphisms, CELLULAR IMMUNOLOGY, section VI:B. The PCR product will give the same band for all samples. To detect polymorphisms, further digest the PCR product with digestive enzymes according to the manufacturers' instructions.
- Run the digested PCR product on 3% agarose gel (use NuSieve GTG) electrophoresis at 80 V and stain the gel with 0.1% ethidium bromide. Visualize the gell under UV light and take a photograph

IX:D. Amplification Refractory Mutation System (ARMS) PCR for detection of previously characterised mutations in cytokine genes

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Equipment

automated thermal cycler
agarose gel electrophoresis
UV-transilluminator

Materials and reagents

Tris–HCl (pH 8.8)
$MgCl_2$
Taq polymerase
dNTP
Tween 20 (ABgene)
primers
ethidium bromide

CAUTION: This chemical is toxic and mutagenic. Wear protective clothing and use only in a chemical fume hood.

PCR master mix:
25 mM Tris–HCl (pH 8.8)
1.5 to 4.0 mM $MgCl_2$
0.25 *Taq* polymerase
200 µM dNTP
0.01% (v/v) Tween 20 (ABgene)

Primer mix:
specific primer mix (10 µM of generic primer and 10 µM of specific primer)

5 µM of positive internal control (10 µM of internal control forward and 10 µM of reverse primers).

Procedure

- Put 100 to 250 ng of each DNA into two tubes or wells.
- Add other PCR reagents as indicated in the master mix above.
- Program the automated thermal cycler according to manufacturers' instructions.
- Use guidelines in CELLULAR IMMUNOLOGY, section VI:B for VNTR polymorphisms to set annealing temperature and extension time according to primer and product considerations. Run the program for 30 cycles.
- Analyze the PCR products by agarose gel electrophoresis followed by ethidium bromide staining and visualisation under UV light.
  Amplification should occur in only one tube if the DNA template contains wild-type sequence or mutation sequence. Heterozygous individuals show amplification in both tubes. A positive control sequence is required to measure that absence of product is not due to PCR failure.

Reference

Perrey C, Turner SJ, Pravica V, Howell WM, Hutchinson IV. 1999. ARMS-PCR methodologies to determine IL-10, TNF-alpha, TNF-beta and TGF-beta 1 gene polymorphisms. Transpl Immunol 7(2):127-128.

X. Haptoglobin phenotyping using PAGE

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Equipment

gel electrophoresis apparatus
gel drier or oven

Materials and reagents

polyacrylamide gel
Tris–HCl
Tris-base
glycine
distilled water
10% ammonium persulphate
TEMED (tetramethyl-ethylenediamine)
haptoglobin
erythrocyte haemolysate
benzidine stain
filter paper

Buffers:

1.5 M Tris–HCl (pH 8.8)
54.5 g Tris-base
150 mL distilled H_2O

0.5 M Tris–HCl (pH 6.8)
6 g Tris-base
60 mL distilled H_2O

10× running buffer
15.5 g Tris-base
72.1 g glycine
500 mL distilled H_2O

Procedures and preparations

- Cast a polyacrylamide gel with separation gel of concentration 4.7% and stacking gel of 2.5%.

	separating gel (4.7%)	stacking gel (2.5%)
30% bis acrylamide	7 mL	1.3 mL
Tris–HCl	11.25 mL	2.5 mL
distilled water	26.7 mL	6.0 mL
10% ammonium persulphate	150 μL	50 μL
TEMED	37.5 μL	18 μL

- Add 3 μL of erythrocyte haemolysate to 10 μL of serum or plasma and control haptoglobin.

- Mix the solution well and incubate it for 5 min to enable haptoglobin in serum to bind to hemoglobin.
- Add 10 µL of loading buffer. Load the sample onto the gel using a template.
- Run electrophoresis at a constant voltage of 80 to 120V using 1× running buffer.
- Stain the gel for 5 to 15 min with benzidine stain.
- Wash the gel in distilled water.
- Transfer the gel onto filter paper and dry it using a gel dryer or oven.

References

Davis I, Orstein J. 1968. Disc electrophoresis, acrylamide gel columns: *In:* Williams, CA, Chase MW, eds. Methods in Immunology and Immunochemistry. Vol. 2. New York: Academic Press; pp. 34-47.

Coligan JE, et al., eds. 2003. Current protocols in immunology. Vol. 2. Electrophorectic separation of proteins. Chapter 8, section III. New York: Wiley-Interscience.

MOLECULAR BIOLOGY

I. DNA isolation from *Plasmodium falciparum*

I.A: Small-scale genomic DNA isolation from *Plasmodium falciparum*

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Equipment

centrifuge (4 °C)
speed-vac
agarose electrophoresis unit
spectrophotometer

Materials and reagents

infected erythrocytes (a few mL with about 10% parasitemia)
phosphate-buffered saline (PBS) (pH 7.2)
5% saponin solution
lysis buffer:
40 mM Tris–HCl (pH 8.0)
80 mM EDTA (pH 8.0)
2% SDS, 0.1 mg/mL
proteinase K (Add the proteinase K just before using the buffer.)
phenol equilibrated with 0.1 M Tris–HCl (pH 7.0)
chloroform
RNase (Stratagene, RNace-It Ribonuclease Cocktail)
3 M sodium acetate (pH 5.0)
absolute ethanol
70% ethanol
TE-buffer:
10 mM Tris–HCl (pH 8.0)
1 mM EDTA (pH 8.0)

Procedure

- Centrifuge the infected erythrocytes at 3,000 × *g* for 2 min. Wash the cells once in cold PBS.
- Resuspend the cells from one microfuge tube (1.7 mL) in 1 mL of PBS.
- Add and gently mix 10 μL of 5% saponin (for a final concentration of 0.05%).
- Immediately centrifuge the tube at 6,000 × *g* for 5 min after lysis is observed.
- Remove the supernatant.
- Add 25 μL of lysis buffer and 75 μL of distilled water to the pellet.
- Incubate the tube at 37 °C for ~3 h with intermittent stirring by hand.
- Add 100 μL of distilled water, then 200 μL of phenol. Mix well and centrifuge at 2,000 × *g* for 8 min.
- Extract likewise with 200 μL of chloroform.
- Add 2 μL of RNace-It cocktail for 30 min at 37 °C.
- Extract with phenol and chloroform as above.

- Precipitate the gDNA by adding one-tenth volume of sodium acetate and 2.5 volumes of absolute ethanol; allow the tube to sit for 2 hours or overnight at −20 °C. The DNA can be stored this way as well.
- Centrifuge the precipitate at 2,000 × *g* for 30 min at 4 °C, wash it gently once with 70% ethanol, dry it in a speed-vac, and gently resuspend the pellet in 25 to 100 μL of distilled water, depending on its size.
- Determine the DNA concentration at OD_{260}; 2 mL of culture may yield ~2 μg of gDNA. Visualize ~0.5 μg on a 0.8% agarose gel to see that the gDNA runs as a high-molecular weight, somewhat broad band and is thus unsheared and free of RNA.

I.B: Preparation of *P.falciparum* genomic DNA.

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see: Transfection, VI.B, page 376

II. RNA extraction from *Plasmodium falciparum*

II:A. RNA extraction from *Plasmodium falciparum* using guanidine thiocyanate and acidic phenol

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Equipment

centrifuge (4 °C)
JA-20 Beckman rotor
small-blade Polytron homogenizer (Brinkmann)

Materials and reagents (see also Solutions below)

PRBC (Check PRBC to determine the parasitemia and rosetting rate. Usually eight 75-cm^2 bottles with a parasitemia of 7 to 10% will yield ~200 to 300 μg of RNA.)
cold sterile PBS (pH 7.2)
complete solution D (with 72 μL 2-mercaptoethanol (2-ME)/10 mL of Solution D or 288 μL of 2-ME/40 mL of Solution D). Remove 1 mL into a microcentrifuge tube; store the rest at 4 °C for *Day 2*. Solution D with 2-ME added is stable for 2 weeks at 4 °C.)
2 M sterile sodium acetate (pH 4)
acidic phenol:chloroform:isoamylalcohol (125:24:1) (Ambion 9722)
ice cold 2-propanol
distilled water
50-mL Falcon tubes (BD Labware)
tubes (see under MOLECULAR BIOLOGY, sections II:D)

Solutions

Solution D for RNA isolation:

100 g guanidine thiocyanate
117.2 mL sterile distilled water
7 mL 0.75 M sodium citrate (pH 7.0) (autoclaved)
10.6 mL 10% N-lauroyl-sarcosine (Sigma L9150; filtered; store stock at room temperature)
Check the pH, which should be ~7.0.
The solution can be stored for 3 months at room temperature.

Working solution:

720 μL 2-mercaptoethanol/100 mL of Solution D; keeps for 2 weeks if stored at 4 °C.

2 M sodium acetate, 50 mL:

Add 8.2 g of anhydrous sodium acetate to about half the final volume of distilled water; as the salt is dissolving, adjust the pH to 4.0 with ~16 mL of concentrated acetic acid; bring to volume and autoclave.

Tris–EDTA buffer (TE) (see also Sambrook et al. 1989):

10 mL 1 M Tris–HCl
2 mL 0.5 M EDTA (pH 8.0)
988 mL water
Combine Tris and EDTA in water; adjust pH to 7.4; autoclave. Store the solution at 4 °C; it is stable for 6 months.

10× electrophoresis buffer (0.2 M MOPS and 10 mM EDTA):

40 mL 1 M MOPS (pH 7.4) (autoclaved; 209.3 g/mol)
4 mL 0.5 M EDTA (pH 8.0) (autoclaved)
156 mL distilled water (autoclaved)

RNA loading buffer, deionized formamide:

25 g mixed-bead ion exchange resin (Bio-Rad, 142-6424)
250 mL of formamide (Sigma F7503)
Wrap an Erlenmeyer flask in foil. Add resin beads and formamide. Stir the mixture for 60 min in the hood. Filter through Whatman No. 1 filter paper. Store aliquots in dark bottles at −20 °C (stable for 1 year).

RNA loading buffer:

3.37 mL deionized formamide
1.08 mL formaldehyde (37%)
0.5 mL 10× electrophoresis buffer
20 μL 0.5 M EDTA (pH 8.0)
285 μL glycerol (sterile)
50 μL 10% SDS
50 μL bromphenol blue, 0.5% (or 5 mg)
50 μL xylene cyanole, 0.5% (or 5 mg)
Aliquot, then store at −20 °C.

Agarose gel (small 40 mL):

0.4 g agarose (Sigma A9539)
29 mL distilled water
Add agarose to sterile water, heat to boiling to dissolve the agarose, then cool to 65 °C.
Add:
4 mL 10× electrophoresis buffer
50 μL 0.5 M EDTA
In the hood, quickly add 7 mL of formaldehyde (or less if using Sigma's buffers). Pour the gel solution into a clean, taped gel tray containing a comb. Remove bubbles and allow the gel to cool.

20× SSC stock (5 L):

876.5 g sodium chloride
441.0 g sodium citrate
Add salts to distilled water and mix to dissolve. Add distilled water to total of 5 liters, adjust the pH to 7.0 with HCl, and autoclave.

Prehybridization/hybridization fluid:
0.5 M Na_2HPO_4 (pH 7)
1 mM EDTA
7% SDS

Probe removal solution
0.1% SDS
Place a nylon filter in the boiling solution and remove from heat. After 15 min remove and check the filter. Either repeat the treatment or simply expose the filter to check that the probe is really removed. Keep the filter moist at all times by placing it in a sealed hybridization bag. It may be stored at −20 °C.

Isolation of RNA, *Day 1*

- Cool the centrifuge for 50-mL Falcon tubes.
- Pool cells (PRBC) into two 50-mL tubes on ice.
- Centrifuge the cells at 1,800 rpm for 5 min at 4 °C.
- Remove the supernatant. Wash the cells with ~30 mL of cold PBS. Gently resuspend the cells. Centrifuge them again.
- Remove the supernatant. Resuspend the cells in cold PBS to ~20 mL/tube and transfer into centrifuge tubes that have lids (i.e., not Corex tubes) and that withstand 15,000 × *g* and organic solvents. Centrifuge them as above.
- Remove the supernatant and set the cells on ice.
- Rinse and clean a small-blade Polytron homogenizer by running distilled water in it, followed by some solution D.
- Add 10 mL of solution D per centrifuge tube and suck up and down to disrupt cells.
- Homogenize the cells on “medium” setting for 15 s. Avoid frothing. Keep the cells on ice.
- Clean the homogenizer by running solution D in it, then running distilled water, with changes of water until no trace of homogenized material appears on the blades.
- To each tube add:
  1.0 mL 2 M sodium acetate (pH 4) (× 0.1 volume)
  10 mL acidic phenol (× 1 volume)
  2.0 mL chloroform:isoamyl alcohol (× 0.2 volume)
- Cap the tubes with lids and vortex well for ~1 min. Set them on ice for 15 to 30 min. Meanwhile cool the centrifuge and rotor (JA-20 Beckman rotor) to 4 °C.
- Invert and vortex to mix the samples, then immediately centrifuge the tubes at 11,000 rpm for 30 min at 4 °C.
- Remove and save the supernatant in two new centrifuge tubes (Nalgene 3110). Use lids. Avoid material from the interphase.
- Add an equal volume of 2-propanol (at −20 °C), mix, and set the sample at −20 °C overnight or at least for a few hours.

Further purification of RNA, *Day 2*

- Cool the JA-20 Beckman rotor. Mark the side of each tube where the “invisible” RNA pellet is expected to be. Centrifuge the tubes at 11,000 rpm for 30 min at 4 °C.
- In a ventilated hood, carefully remove the supernatant with a 10-mL pipette, being careful to go down the opposite side of the tube from where the pellet is expected. There should be an almost transparent, gel-like pellet. Mark the tube where the pellet is.
- Dry the tubes some in the hood, but do not over-dry them.
- Resuspend both tubes’ RNA contents in a total of 0.5 mL of Solution D.
- Transfer the contents into a microcentrifuge tube. Precipitate it with an equal amount of 2-propanol overnight at −20 °C (or at least for a few hours).

References

Chomczynski P, Sacchi N. 1987. Single-step method of RNA isolation by acid guanidinium thiocyanate–phenol–chloroform extraction. Anal Biochem 162(1):156-159.

Sambrook J, Fritsch EF, Maniatis T. 1989. Molecular cloning: a laboratory manual. 2nd ed. Cold Spring Harbor, New York: Cold Spring Harbor Laboratory Press.

II:B. Small-scale RNA extraction from *Plasmodium falciparum* using Trizol solution

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Equipment

centrifuge (4 °C)

Materials and reagents (see also Solutions below)

Trizol reagent (invitrogen/ambion)
Chloroform
acidic phenol:chloroform:isoamylalcohol (25:24:1) (Ambion 9722)
ice cold isopropanol
ice cold 70% ethanol in DEPC water
nuclease-free water
sterile syring with 0.6mm needle (optional)

Protocol

- Tranfer 50-100ul of packed infected RBC >5% parasitemia into an autoclaved eppendorf tube.
- Add 1ml Trizol solution.
- Homogenize the RBC by pipetting or by passing through the needle several times, do not vortex.
- Incubate at room temperature for at least 5 mins.
- Add 200ul chloroform (0.2 vol of Trizol).
- Shake very vigorously for 15 sec, do not vortex.
- Let to stand for 3 mins.
- Spin at 12000 g for 15 mins in pre-chilled centrifuge at 4°C.
- Recover the upper aqueous layer (there will be ~600ul but take only 550ul so to avoid the interface).
- Add an equal volume of acidic phenol solution.
- Repeat from the shaking step, but recover only 500ul this time after centrifugation.
- Add 0.5ml of ice cold isopropanol and incubate for at least 10 mins at room temperature.
- Spin the tube at 12000 g for 10 mins in pre-chilled centrifuge at 4°C.
- A small pellet is usually visible for yield >1ug
- Remove as much supernatant as possible
- Add 1ml ice cold 70% ethanol
- Spin at 7500 g for 5 mins in pre-chilled centrifuge at 4°C.
- Remove as much supernatant as possible and dry the pellet briefly (if most supernatant is removed, drying will not take more than 3 mins).
- Resuspend in desired amount of nuclease-free water and heat the samples at 55°C for 10 mins.
- Store at at -80°C.

Notes to the above procedure:

1: Use needle if working on later stages or if the parasitemia is very high.

2: Do not vortex at anytime, it may jeopardize the integrity of the RNA and sheer the DNA into smaller fragment increasing the risk of contamination.
3: Check purity of RNA by looking at the absorbance ratio of 260/280nm, this reading indicates protein contamination and normally gives reading >2, but it may be lowered to 1.6 usually when the total RNA yield is low. The absorbance ratio of 260/230nm indicates other contamination, such as residual phenol. A good reading should normally be >2, it may come down drastically if the washing or if the supernatant is not completely removed. Re-precipitation and washing maybe needed if the reading is too low. As phenol contamination will affect many downstream protocols, such as cDNA synthesis, qPCR and RNA-seq.
4: Bio-analyzer can be used for assessing DNA contamination and RNA integrity. Perform DNase treatment only when there is suggestion of a lot of DNA contamination, otherwise small contamination can be accounted for by using proper controls in many downstream applications. Several different commercial DNase kits seem to have non-specific activity against RNA as well when we analyzed with Bio-analyzer.

II:C. Measurement of RNA yield and purity, and visualization on agarose–formaldehyde gel

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Equipment

microfuge

Materials and reagents

premixed RNA electrophoresis buffer (Sigma M5755)
RNA gel loading buffer (Sigma R4268)

10× electrophoresis buffer:
23.4 mL distilled water
600 μL 0.5 M EDTA (pH 8)
6 mL 1 M MOPS (pH 7.4)
This makes 30 mL total, approximately the amount needed for a regular small gel system.

formaldehyde
agarose
1 M EDTA (pH 8.0)
distilled water (or DEPC-treated water)
3 M sodium acetate (pH 5.2)
ethidium bromide
CAUTION: This chemical is toxic and mutagenic. Wear protective clothing and use only in a chemical fume hood.
bromphenol dye

Procedure

- Extract and purify the RNA as instructed in MOLECULAR BIOLOGY, section II:A, *Days 1* and *2.*

Day 3:

- Microfuge the RNA for 30 min at 12,000 to 14,000 rpm and 4 °C.
- Meanwhile prepare buffers and gel. Use EITHER:
  premixed RNA electrophoresis buffer (Sigma M5755) and RNA gel loading buffer (Sigma R4268). The gel should then contain a lower amount of formaldehyde of 2.4%.
  OR:
  10× electrophoresis buffer
- Have formaldehyde ready in the hood.
- Prepare a 1% agarose gel:
  0.4 g agarose
  29 mL of distilled water
  Mix, heat, and cook briefly. Cool gel liquid some. Then add:
  4 mL 10× electrophoresis buffer
  25 μL 1 M EDTA (pH 8.0)

- Bring the gel liquid to the hood, add 7 mL of formaldehyde, mix, then quickly pour the gel. Use a comb which is set aside for RNA-work so that no RNases from fingers are present on it.
- Allow the gel to solidify in the hood, but do not over-dry.
- Continue with the RNA sample. Wash precipitated RNA 2 times with 900 μL of 70% ethanol at −20 °C . Allow it to dry some in the hood.
- Set a small water bath to 60 to 65 °C.
- Dissolve the RNA in ~100 μL of autoclaved distilled water (or DEPC-treated water) depending on pellet size. If the RNA does not dissolve well with pipetting, heat it briefly at 65 °C. Pipette to dissolve, then keep the RNA on ice.
- Use ~1:100 dilution of RNA for spectrophotometric measurement, preferably in a microcuvette to minimize the amount of RNA which is lost to absorbance measurements.
- Use the program for fixed wavelength at 260 and 280 nm.
- Take absorbance readings after blanking with distilled water.
- Calculate:
  RNA concentration of stock: (Abs) (dil.) (40 μg/mL)/1000 = RNA μg/μL
  total yield: (above #) (μL stock RNA) = μg RNA
  purity: Abs_{260}/Abs_{280} = should be ~1.8 to 2.0 if not too much protein contamination is present.
- Take out 5 μg or less of RNA to run in a lane on the gel.
- The RNA left to precipitate is: total yield – spec. reading – gel loading.
- Precipitate the RNA left by adding one-tenth volume of 3 M sodium acetate (pH 5.2), mix, then add 3 times volume of −20 °C absolute ethanol. Store RNA at −70 °C.
- If RNA volume taken out for gel is >10 μL, speed-vac it down to dryness. Otherwise add RNA loading buffer to 19 μL (or less if using Sigma's premade solution, see manufacturer's suggestions). Mix and heat for 5 to 10 min at 60 to 65 °C. Set on ice
- Add 1 μL of ethidium bromide (at ~250 ng/mL); mix. Pour 1× electrophoresis running buffer over the gel and load.
- Run the gel at ~50 V for more than 1 h with the bromphenol dye running about half way. Use commercial RNA marker(s) if needed (i.e., Gibco). For best results the gel should be run at 4 °C. View and photograph the gel under UV-light. Two RNA bands should be quite visible corresponding to rRNAs. A third low rRNA band may at times also be visible; it is usually fuzzy. After a distilled-water rinse, the gel may be transferred to a nylon filter and be Northern probed using manufacturer's protocols.

References

Chomczynski P, Sacchi N. 1987. Single-step method of RNA isolation by acid guanidinium thiocyanate–phenol–chloroform extraction. Anal Biochem 162(1):156-159.
Sambrook J, Fritsch EF, Maniatis T. 1989. Molecular cloning: a laboratory manual. 2nd ed. Cold Spring Harbor, New York: Cold Spring Harbor Laboratory Press.

II:D. Reliable RNA preparation for *Plasmodium falciparum*

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Equipment

benchtop centrifuge, refrigerated
microfuge, ambient or refrigerated
water baths/heating blocks (37 °C and 60 °C)
spectrophotometer/cuvettes for reading absorbance at 260 nm and 280 nm (optional)

Materials and reagents

parasite-infected red blood cells (iRBC):
0.5 mL packed cell volume at 10% parasitemia, expected yields:

rings	50–150 μg total RNA
trophozoites, schizonts, gametocytes	250–750 μg total RNA

gloves
TRIzol Reagent: (Invitrogen) 15596-026 (Follow manufacturer's suggestions for safety and storage.)
chloroform (AnalAR from BDH, or Sigma C5312, or similar)
2-propanol (AnalAR from BDH, or similar)
formamide (molecular biology grade, Sigma F9037, or similar. It is not necessary to deionize before use. Open bottle in chemical fume hood.)
RNAse-free, chloroform/phenol-resistant plastics
screw-top test tubes*
1.5-mL microfuge tubes*
pipette tips*
**Straight from manufacturer is usually ok; autoclave if not sterile, taking care to wear gloves in handling.*

optional:
RNAse-free water (Sigma W4502 or Milli-Q purified or similar)
RNAse-free 3M sodium acetate solution (pH 5.2) (Sigma S7899 or similar)
Either buy RNAse-free water and sodium actetate solution or treat distilled water and sodium acetate solution with 0.1% diethyl pyrocarbonate (DEPC; Sigma, D5758) as described in Sambrook et al. 1989; p. 7.3; this requires autoclaving.
RNAse-free 75% ethanol/25% RNAse-free water
RNAse-free absolute ethanol (AnalAR from BDH, or Sigma E7023, or similar)

Protocol

Key RNA advice: Wear gloves for everything, and use RNAse-free plastics.

Preserve RNA in TRIzol

- Spin cells gently at 1,800 rpm (600 × *g*) for 4 min at room temperature. *(example: 0.5 mL of packed infected RBC, about 10% parasitemia*)[1,2]
- Remove supernatant (s/n)[3]. Tap the tube to loosen the cell pellet[4].

- Add TRIzol, prewarmed to 37 °C[5].
  For RINGS: add 10 pellet volumes of TRIzol.
  (example: 5 mL of TRIzol to 0.5 mL cells)
  For all other stages: add 20 pellet volumes of TRIzol.
  (example: 10 mL of TRIzol to 0. 5 mL cells)
- Shake the sample to dissolve any clumps.
- Incubate the sample at 37 °C for 5 min.
- Either store the sample at −70 to −80 °C[6] or continue.

EXAMPLE
Beginning with
0.5 mL of iRBC,

to the rings *(trophs)*
↓
add TRIzol
5 mL *(10 mL)*
↓
and chloroform
1 mL *(2 mL).*

Sample size is now:
3 mL *(6 mL).*

Add 2-propanol
2.5 mL *(5 mL).*

Continue extraction and precipitate RNA

- (Thaw samples at 37 °C, if necessary.)
- Add 0.2 TRIzol volumes of chloroform.
  (example: sample in 5 mL TRIzol, add 1 mL of chloroform)
  (example: sample in 1 0 mL TRIzol, add 2 mL of chloroform)
- Vigorously shake the sample; let it stand at room temperature for 2 to 3 min.
- Spin the tube at 4 °C for 30 min (microfuge = maximum 9,000 × *g*; or for 15-mL tubes in benchtop centrifuge, 1,000 to 1,400 × *g* is fine)[7].
- Remove the aqueous (top, clear, RNA-containing) layer to a new tube[8]. Expect to recover up to 0.6 TRIzol volumes, but avoid the interface!
  (example: from sample in 5 mL of TRIzol, expect 3 mL of aqueous layer)
- Add 0.5 TRIzol volumes of 2-propanol to the RNA; invert the tube several times to mix.
  (example: to 3 mL of aqueous layer, add 2.5 mL 2-propanol)
- Transfer/split large samples to 1.5-mL snap-cap microfuge tubes.
- Precipitate the samples for AT LEAST 2 h (up to several days) at 4 °C.

Finish preparation, check quantity/ quality of RNA

- Spin the sample at 12,000 × *g* (13,000 to 14,000 rpm, microfuge) for 30 min at 4 °C or room temperature[9].
- Remove as much as possible of the supernatant[10] (*Optional 75% ethanol wash[11]).
- Air dry the sample, inverted, at room temperature, for no more than 5 min.
- Add formamide (usually a total of 100 μL for a starting pellet of 0.5 mL of infected RBC)[12].
- Heat the sample at 60 °C for 10 min; place it on ice.
- Resuspend the pellet by pipetting[13].
- Check the absorbance at 260 nm[14].
- Store the pellet at −70 to −80 °C.

Notes to the above procedure:

5: Especially for rings, cells pellet much more efficiently in 15-mL tubes than in 50-mL tubes. Subsequent steps are also easiest in 15-mL tubes, so harvest large volumes of cells directly in 15-mL tubes.

6: Use phenol/chloroform-resistant tubes.

7: After removing tubes, adjust centrifuge temperature down to 4 °C for the next spin.
8: Do not wash cells or lyse them with saponin. It is important to lyse the infected red blood cells directly in TRIzol as soon as possible after removing them from culture.
9: TRIzol seems to work best if it is prewarmed to 37 °C. Make sure lids are tightly sealed before shaking the tubes. Do the 37 °C incubation *before* freezing the samples to ensure complete complexing of nucleoproteins. The TRIzol can cope with extracting the RNA from hemoglobin, of which there is obviously plenty. TRIzol does not cope with separating RNA from DNA very well. Trophozoites/late stage parasites synthesize lots of DNA, and therefore require excess TRIzol.
10: Because the RNA is very stable at this point (if stored at −70 to −80 °C), collecting lots of samples is easy. Store them until you can process them all at once.
11: Do NOT spin at room temperature. If using a microfuge, use screw-cap tubes. Snap-cap tubes tend to leak and make a horrible mess of the inside of the centrifuge.
12: Avoid the interface to avoid DNA contamination. It is easier to recover more of the aqueous layer from 15-mL tubes than from 50-mL tubes. The DNA seems to collect right *above* the interface, so do not try to collect every last microliter of aqueous layer. The manufacturer's protocol for retrieving DNA from this does not seem to work due to massive amounts of hemoglobin. If anyone manages, please let me know!
13: Give tubes a quick vortex before spinning. Place tubes in the microfuge with hinges pointing outwards (for future identification of RNA pellet). Room temperature spin is fine, but 4 °C is ideal.
14: Remove supernatant in two stages:
    a. Keeping pipette tip away from the putative pellet (directly beneath hinge, at bottom of tube), remove nearly all of the supernatant. Save the supernatant to a new tube if you are worried about losing your sample.
    b. Give the tube a quick 'flick spin', again with hinges pointing outwards. This gets all those dribbles on the side of the tube down to the bottom again. Remove last bits of supernatant, as much as possible, taking care to avoid touching the very tiny, glassy pellet. If there is a large pellet, there is probably DNA or protein in it.
15: At this point, it is optional to add a 75% ethanol wash step: Add 0.5 mL of 75% ethanol: 25% DEPC–water, ice-cold; spin for ~5 min in a microfuge, and remove all supernatant. This gets rid of all traces of phenol and is particularly useful for cDNA preparations (see next note).
16: Add the formamide to the tube, avoiding touching the 'pellet'. Split the total formamide volume among the microfuge tubes if it was a large sample split to several tubes. RNA is very stable for a long time in formamide. However, the formamide affects downstream enzymatic applications. If you plan to make cDNA, it is worth considering resuspending the RNA pellet in DEPC–water, if you have it. If you have RNA samples in DEPC–water, store them in aliquots at −80 °C, but beware that the RNA is not very stable in water, and does not survive many freeze-thaw cycles. One way around this is to precipitate the RNA (add one-tenth volume of RNase-free 3M sodium acetate [pH 5.2], and 2.5 volumes of ethanol), then store at −80 °C. This is very stable, and will last 'forever'. When

you need some RNA, just thaw the tube, vortex it, and remove the volume necessary to give the desired amount; spin it down in the microfuge for ~30 min.

17: By pipetting up and down, mix each sample thoroughly, making sure that nothing is stuck to the bottom of the tube. The pellet should have completely dissolved. Pool tubes of the same sample-type back together at this point. Set aside small volumes for measuring absorbance or running on gel.

18: Spectrophotometric measurement of quantity:

Check absorbance at 260 nm: Try 1 μL in 500 μL of water, remembering to blank against 1 μL of formamide in 0.5 mL of water.

Conversion: OD 1 = 40 μg RNA

Compare to OD 280. The ratio OD260/OD280 should be close to 2.0 for pure RNA. If there is any contaminating phenol, this number will be lower and the quantification will not be accurate.

If this of great concern, at the point marked *above, add the 75% ethanol wash step. It does not seem to make much difference, but gives some people peace of mind that the RNA is really clean.

Agarose gel measure of quality:

Using the procedure outlined for Northern blots below (see MOLECULAR BIOLOGY, section II:D), run a small gel, with small lanes. Run 1 to 2 μL of each sample.

There should be two main ribosomal bands and a few minor bands of unknown provenance. Good RNA preparations have roughly equal amounts of both main ribosomal bands. If the RNA is degraded, there will be a 'comet tail' and no discrete bands. If there is plenty of DNA, there will also be a faint smudge close to the wells. Sometimes this does not matter, but sometimes it is really annoying. There are methods for getting rid of the DNA, but they are expensive and not fun.

Finally, if your sample had human white blood cells in it, you'll see more than two ribosomal bands!

References

Kyes S, Pinches R, Newbold C. 2000. A simple RNA analysis method shows *var* and *rif* multigene family expression patterns in *Plasmodium falciparum.* Mol Biochem Parasitol 105(2):311-315.

Sambrook J, Fritsch EF, Maniatis T. 1989. Molecular cloning: a laboratory manual. 2nd ed. Cold Spring Harbor, New York: Cold Spring Harbor Laboratory Press. pp 7.3-7.4.

TRIzol is based on the following:

Chomczynski P, Sacchi N. 1987. Single-step method of RNA isolation by acid guanidinium thiocyanate–phenol–chloroform extraction. Anal Biochem 162(1):156-159.

II:E. Simple Northerns

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*NO DEPC-treatment anywhere! **Just wear gloves for everything.***

Equipment

agarose gel tank, combs, and power pack
deep plastic trays for soaking gels (rinsed with 1× distilled water)
UV transilluminator and gel photography equipment
heating block/water bath (60 to 65 ^{o}C)
ice to chill samples

Materials and reagents

gloves
hydrogen peroxide, 30% (Sigma H1009)
deionized water (or 1× or 2× distilled water), but not autoclaved or DEPC-treated, for all gel buffers and solutions, except as noted*.
No solutions are autoclaved

1× TBE (as for DNA gels):
0.089 M Tris
0.089 M boric acid
2 mM EDTA

5× TBE:
54 g Tris-base
27.5 g boric acid
20 mL 0.5 M EDTA
Combine ingredients; makes 1 liter.

agarose (normal grade as for DNA gels)
1 M guanidine thiocyanate (Sigma G6639; MW=118.2); i.e., 118 mg/mL of sterile* water (non-DEPC, as used for DNA work is fine)
Make only as much as you need on day of use (e.g., ~0.5 to 0.6 mL).
TOXIC; be sure to dispose of waste properly.

DNA loading dye containing bromophenol blue
formamide (Sigma F9037, molecular biology grade).
Not necessary to deionize. Open in chemical fume hood.

fresh, dilute ethidium bromide solution (~0.1 to 0.5 μg /mL, ~200 mL)
CAUTION: This chemical is toxic and mutagenic. Wear protective clothing and use only in a chemical fume hood.

fresh 7.5 mM NaOH, ~1 to 1.5 liter (fresh, made on same day)
for blotting (see Sambrook et al.)
Hybond N+ (Amersham)

Whatman 3M paper
paper towels
2 glass plates, one big enough to fit over a shallow plastic tray
shallow plastic tray
2× SSC:
0.3 M NaCl
0.03 M sodium citrate
(1 liter of 20× SSC = 175.3 g NaCl, 88.2 g sodium citrate, to pH 7 with HCl; dilute 1:10 for 2× SSC)

Protocol

Electrophoresis of RNA and capillary transfer to nylon membrane

- Clean gel apparatus by soaking everything including combs in 3% H_2O_2 for 10 min (1:10 dilution of 30% stock in 1× distilled H_2O)
  or in 0.5 M NaOH for 2 h,
  or in 1% SDS for 2 h
  then rinsing in 1× distilled H_2O (which is not necessarily sterile or RNAse-free!).
- For a 100-mL, 1% agarose gel, using a relatively clean bottle, combine 100 mL of 1× TBE + 1 g agarose, and heat, with caution, in a microwave oven .
  The best resolution depends on the expected transcript size. Use the following guide for agarose concentration:
  0.8% agarose for large transcripts (greater than 7 kb)
  2% agarose for short transcripts (1.5 to 3 kb)
- Cool the gel to 55 to 60 °C. Check this with a relatively clean thermometer.
- Add 0.5 mL of freshly-made 1 M guanidine thiocyanate (final concentration = 5 mM G-SCN).
- Pour the gel and let it set for at least 30 min at room temperature.
- Prepare the samples while the gel sets.
  amount of RNA:
  For 0.8% agarose gel, use 6 to 10 μg of total RNA per lane.
  For 2% agarose gel, use 3 to 5 μg per lane.
  volume of RNA to load:
  total, approximately 5 to 20 μL per lane
  loading dye:
  Not necessary if samples are in formamide.
  If your samples are in water, add an equal volume of formamide (final 50% formamide or greater).
  NOTE: Don't put formaldehyde in your samples. It does not work on this type of gel!
  If you absolutely need loading dye, try formamide with final 1× TBE, 0.5% bromophenol blue. Add this AFTER denaturing your RNA in formamide.
- Denature ~2 μg of size-standard RNA, in ~10 μL of formamide for ~10 min; place it on ice.
- Denature samples at 60 to 65 °C for 2 min; place it on ice.
- Having rinsed the gel tank, place the set gel in it, pour in 1× TBE to cover it, then remove the comb and any end blocks.
- Load the gel. Load a blank lane with DNA loading dye between the size marker and the samples to track the running of the samples. This also keeps your

marker from spilling into a sample lane, which sometimes makes a mess of hybridizations.
- Electrophorese samples into the gel for approximately 10 to 15 min at 110 volts, then run at 70 to 80 volts (3.5 v/cm) until the bromophenol blue is approximately 8 to 10 cm from the well. The process takes about 3 to 4 h.
- Stain the samples in fresh, dilute, ethidium bromide solution for ~10 min.
- Destain the for a few minutes in 1× TBE using buffer left over from the gel box.
- Photograph them with a ruler next to the gel. Leave the gel on UV transilluminator for ~2 min. This nicks the RNA, helping with transfer of larger transcripts.
- Soak the gel in ~200 mL of 7.5 mM NaOH, twice, for 10 min each time.
- Set up the capillary transfer to Hybond N+ in 7.5 mM NaOH to run overnight (not less than 8 h, especially important for large transcripts). (See Sambrook et al. 1989.)

Disassemble blot and start hybridization
- Neutralize the filter in 2× SSC for 5 min.
- Air dry the filter. Put the filter on UV light box at this point and draw size marker positions directly on the filter with a pencil.
- UV-crosslink, if you have a cross-linker, but this is not necessary.
- Prehybridize and hybridize as usual (see Kyes et al. 2000 for solutions and conditions). To start, try 55 ^{o}C hybridization, 60 ^{o}C wash in 0.5× SSC/0.1% SDS.

References

Goda S, Minton N. 1995. A simple procedure for gel electrophoresis and northern blotting of RNA. Nucleic Acids Res 23(16):3357-3358.

Kyes S**,** Pinches R, Newbold C. 2000. A simple RNA analysis method shows *var* and *rif* multigene family expression patterns in *Plasmodium falciparum.* Mol Biochem Parasitol 105(2):311-315.

Sambrook J, Fritsch EF, Maniatis T. 1989. Molecular cloning: a laboratory manual. 2nd ed. Cold Spring Harbor, New York: Cold Spring Harbor Laboratory Press; pp. 7.46-7.49.

III. *Plasmodium falciparum* cDNA library construction

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Equipment

centrifuge (4 °C)
agarose electrophoresis unit
50-mL Falcon tubes (BD Labware)
Polytron homogenizer (Brinkmann)
centrifuge (Beckman)
speed-vac

Materials and reagents

Consider using kits from Stratagene for extraction of RNA and construction of cDNA libraries. They are generally more commonly employed than the Pharmacia kits used below. However, we did successfully make several cDNA libraries from two different strains using the Pharmacia kits.

Throughout the following protocols (MOLECULAR BIOLOGY III:A–E), there are references to the appropriate protocols from the companies from which library materials were bought. Further changes in these general "company-protocols" have been outlined below to facilitate *Plasmodium falciparum* library construction.

III:A. Cell preparations at desired cell stage(s)

Grow parasites in 75-cm^2 large flasks (12 mL per flask). You will need several sets of large flasks to be able to purify a workable amount of mRNA. We usually used 8 bottles/preparation where 8 bottles with ~10% parasitemia would yield 100 to 200 μg (sometimes even 300 μg) of total RNA. If rosetting parasites are desired, use parasites with ≥ 50% rosetting rate. See also protocols on cell culturing in PARASITES, section I, and synchronization of erythrocytic stages if that is desired (PARASITES, section IV).

III:B. RNA preparation (total RNA)

See separate protocol for RNA preparation in MOLECULAR BIOLOGY, section II above. Store RNA as a precipitate in 3× volume of ethanol with 0.3 M sodium acetate at −70 °C in microfuge tubes.

III:C. mRNA selection of pooled total RNA

See general protocol from 5Prime → 3Prime for mRNA isolation using oligo(dT) cellulose spin columns. (See also other companies for smaller amounts of mRNA isolation.)

Procedure

- Centrifuge microcentrifuge tubes with RNA in microfuge at 4 °C for 30 min.
- Wash samples with 70% cold ethanol and centrifuge them again for ~20 min.
- Dry tubes upside down in a hood with good airflow or in a speed-vac.

- Add 50 to 100 μL of pure "kit-H_2O" to each tube and resuspend the RNA. Pool the RNA into one tube.
- Heat the sample at 65 °C for a few minutes, then place it on ice.
- Load 1 to 5 mg of total RNA per column. See company protocol (5Prime → 3Prime) for the rest.
  Examples of mRNA yields:
  One sample of ~2 mg total RNA gave:
  ~ 87 μg mRNA after the 1st selection (~3.5%)
  ~ 61 μg mRNA after the 2nd selection (~2.5%)

  Another sample of ~1.05 mg total RNA gave:
  ~71 μg mRNA after the 1st selection (~6.8%)
  ~42 μg mRNA after the 2nd selection (~4%)

  From gel analysis it was clear that a second mRNA selection is not necessary since no rRNA bands were shown after one round of selection.
  mRNA was stored as a precipitate according to the protocol with muscle glycogen, sodium acetate, and ethanol.

  Store pure mRNA in aliquots of 1 μg, 5 μg, and maybe more.
  Run a few microgram-per-lane samples to visualize mRNA as a faint smear in the range of the two larger rRNA bands. The front of these two bands should however be sharp if unselected total RNA is also run in a separate lane along with mRNA to indicate lack of degradation. See MOLECULAR BIOLOGY, section II:D for RNA-gel protocol. If using pure mRNA for a gel, you may want to blot and fix it to nylon for possible later use in a Northern.

III:D. cDNA synthesis

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Pharmacia's kits: Timesaver cDNA Synthesis Kit was used with some components replaced by the Directional Cloning Toolbox.

Additional reagents needed are indicated in the protocol booklet. If one has good quality distilled water, there is no need to use DEPC which is very hazardous (and can be destructive to RNA if it remains after autoclaving DEPC-treated water).

One needs to have access to the following water bath temperatures:

12 °C overnight (next day at 16 °C)
room temperature
37 °C
65 °C

Optional: dry ice for dry ice/ethanol bath.

Procedure

- Cool the lyophilizer trap and have spin columns ready in autoclaved, sterile Corex tubes.
- Use 4 μg of very pure mRNA per cDNA synthesis (both for FCR3S1 and TM284 strains). One could use 5 μg to possibly increase the recombinant phage.
- Centrifuge the mRNA in an Eppendorf microfuge for 30 min at 4 °C, then wash it gently once with 70% ethanol to remove salts from the small white mRNA pellet. Centrifuge it again for 20 min, then dry it briefly.
- Follow Pharmacia's general protocol; use 1 mL of *Not*I dT_{18} primer from the Directional Cloning Toolbox as the cDNA primer. (See the Pharmacia protocol.)
- Where phenol/chloroform extraction is indicated, use 50 μL of each, followed by an extra step of pure chloroform extraction.
- Ligate the adaptor for 2 to 2.5 h at 16 °C.
- Digest the adaptor with *Not*I for 1.5 h (not 1 h), then extract it again with chloroform/phenol and pure chloroform.
- Precipitate the cDNA in an ethanol/dry ice bath for 30 min.
- If one uses two purification steps with organics (i.e., phenol/chloroform, then chloroform), as opposed to one combined step of phenol/chloroform, one loses more cDNA material.
- It may be appropriate to use 20 μL of column effluent with 2 μg of vector to do the ligations, rather than the 15-10-5 μL scheme suggested in the protocol, since 15 μL of effluent gave more recombinant phage than 10 μL, which gave more than 5 μL. One strain did not yield any recombinant phage with 5 μL of column eluate.

III:E. cDNA insertion, propagation, and amplification in phage using Pharmacia's λ ExCell *Not*I/*Eco*RI /CIP kit

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See Pharmacia's Timesaver cDNA Synthesis Kit protocol along with comments for cDNA and phage ligations. Use the phage λ vector to set up and do ligation reactions with *Plasmodium falciparum* cDNA.

Use extra microfuge spin times (20 min, not 10 min), 70% ethanol washing, centrifugation, and drying of DNA. cDNA/phage may be heated for a few minutes at 50 °C to sufficiently resuspend the cDNA before ligations are done. Incubate ligation reactions for 30 to 40 min at 16 °C, then at 4 °C overnight.

Use Stratagene's Gigapack II packaging extracts to package ligated products into phage. (Now there is the Gigapack III kit, which is easier to use).

Use 3 to 4 μL of the cDNA-λ ligation per packaging. See the Gigapack II protocols.

Control packagings are not done. It is imperative to work very quickly once the extracts are removed from −70 °C to thaw. Package for 1 h 45 min.

Procedure

- Use *Escherichia coli* strain NM522 as the host for phage grown on special minimal plates (see Pharmacia protocols). Inoculate a few colonies in 10 to 20 mL of Luria broth (LB) with 0.2% maltose and 10 mM $MgSO_4$.
- Shake the inoculum at 30 °C overnight.
- The next day, centrifuge the cell suspension at ~2,000 rpm for 8 min and remove the LB medium.
- Resuspend the cells in 10 mM $MgSO_4$ for an OD_{600} of 0.5.
- Cool the cells at 4 °C until use; use within 5 days with phage.
- Restreak stock plates of NM522 about every 3 weeks. Freeze extra bacterial stock at −70 °C as described in the manual using ~20% glycerol in the medium.
- Titer these primary libraries, 3 primary libraries/strain since there were 3 ligation mixtures set up for each strain. Phage dilutions of 1:10 and 1:100 of the primary libraries are recommended to titer.
- For the 1:10 dilution titering, mix 1 μL of phage with 9 μL of SM-buffer (see Stratagene's picoBlue Immunoscreening kit protocol).
- Make another such serial 1:10 dilution to achieve 1:100 phage dilution.
- Of each dilution, mix 1 μL with 200 μL of NM522 bacteria in 10 mM of $MgSO_4$ (see above) and place at 37 °C for ~15 min.
- Add 3 mL of LB low-melt agarose (at 49 °C) to the mixture and pour it on a prewarmed LB plate.
- Incubate the plate at 37 °C overnight.
- About 100 to 250 phage may be visible on the lower dilution plates. Approximately 0.5×10^6 to 1×10^6 independent clones may be generated per "ligation-library".

Amplification of primary libraries

- One needs about 40 to 50 large petri dishes with LB agar (poured fresh 1 to 2 days previously and evenly on a FLAT surface) for a library amplification (~13-cm plates). Each plate can take 20,000 to 40,000 phage; maybe 25,000 is a good number. Follow the rest of the Gigapack protocol for phage amplification.
- Allow phage plaques to grow for 7 to 8 h until they are pinhead sized (not too large).
- Overlay the plates with 9 to 10 mL of SM buffer overnight. It is very important that the plates lie very flat so that there is liquid on the whole plate into which the phage can elute. If the plates don't lie flat, parts will dry up as the buffer soaks into the agar, thus causing some uneven amplifications of phage populations.
- Recover and pool about 7 mL of SM-buffer/plate into 1-liter sterile bottles.
- Remove the bacteria with chloroform as described in the protocol, and aliquot the phage suspension into sterile 50-mL Corex tubes for centrifugation. Freeze most of the amplified libraries as aliquots at −70 °C with some chloroform/DMSO as described.
- Test both primary and secondary libraries for blue/clear phage indicating ratios of phage with no cDNA insert to those with an insert. Those without an insert should have the *LacZ* gene intact and thus produce β-galactosidase that in the presence of inducer (IPTG) and substrate X-gal would form blue plaques. Phage with insert are clear.
- Mix 1 μL of unamplified phage or 5 to 8 μL of a 10^5-dilution of amplified library with 200 μL of bacteria to get 200 to 300 phage plaques per plate.
- Incubate the phage and bacteria for ~20 min at 37 °C, quickly mix with 3.0 mL of 49 °C top agarose, 7.5 μL of 1 M IPTG, and 50 μL of a solution of 250 mg of X-gal per mL of dimethylformamide (DMF), and pour on a prewarmed LB plate.
- Allow the top agarose to harden without a lid for 5 min, then incubate the plate upside down overnight at 37 °C.
- Remove the plates the following day and place them at 4 °C for the day (or alternatively longer) for the blue color to fully develop. The primary libraries have 2 to 3% of phage that are blue (dark or light blue) indicating no insert. The rest should contain inserts. Amplified libraries are only 3 to 5% nonrecombinant.
- For initial titering of primary libraries, 1 μL of 10-fold dilutions is enough to use (see above).
- Dilute amplified libraries in steps of 1:100, where 10 μL of 10^{-6}, 10^{-7}, 10^{-8} (and possibly more) are plated and a range of about 800 down to 50 phage are seen on the increasingly diluted plates.

Examples of titres achieved:

FCR3S1 library: ~3^9 to 10^9 pfu/mL; TM 284 was at ~9^9 to 10^9 pfu/mL.
Libraries were aliquoted as 1-mL samples in microfuge tubes and stored with 50 μL of chloroform, along with some larger aliquots stored in screw-cap glass tubes with some chloroform at 4 °C for library screening. The rest were kept for long-term storage as aliquots in 15-mL tubes with DMSO/chloroform at −70 °C (see also the Gigapack protocol).

Testing excision of inserts from libraries

To get an idea of insert sizes in the libraries, we used recombinant phage directly from the libraries to excise, or release, clones as phagemids. Best results were obtained with

pExCell release directly from the library as opposed to plaque-purified phage clones. For the in vivo excision, we used NP66 *E. coli* accompanying the kit as well as the specified medium and temperature shift from 32 to 39 °C (See Pharmacia λExCell protocol).

- Pick colonies the following day.
- Incubate them in LB-amp overnight.
- Perform plasmid mini-preps using the alkaline lysis method from “Current Protocols” with suggested modifications, such as phenol extraction, etc. (Do not use boiling mini-preps.)
- Use *Pvu*II enzyme to cut around insert sites. Use about half of the digest on a gel. *Pvu*II-cut sites are ~380 bp apart on the vector (i.e., without insert).
- The vector arms should appear as one band of ~2.5 kb. Insert sizes from a few random clones tested were seen from ~0.5 to 2.2 kb. Or simply test insert size by PCR using vector arm primers.

Protocols from the following companies' kits:

5Prime → 3Prime's Maxi oligo (dT) Cellulose spin column for mRNA purification
Pharmacia's Timesaver cDNA Synthesis
Pharmacia's Directional Cloning Toolbox
Pharmacia's λ ExCell NotI/EcoRI/CIP
Stratagene's Gigapack Packaging Extracts
Stratagene's picoBlue Immunoscreening

References

Ausubel FM, et al., eds. Current protocols in molecular biology. New York: Wiley-Interscience; 1988-1999.

IV. PCR of AT-rich genomes/regions

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Noncoding regions of *Plasmodium falciparum* tend to be extremely rich in AT content (>90%). PCR amplification of these regions is significantly improved by lowering the *Taq* polymerase extension temperature from 72 to 60 °C. The other parameters do not need to be changed.
Note: The AT content of the entire genome is estimated to be ~82% while coding regions are thought to be 60 to 70% AT.

References

Bowman S, Lawson D, Basham D, Brown D, Chillingworth T, Churcher CM, Craig A, Davies RM, Devlin K, Feltwell T, et al. 1999. The complete nucleotide sequence of chromosome 3 of *Plasmodium falciparum*. Nature 400(6744):532-538.
Su XZ, Wu Y, Sifri CD, Wellems TE. 1996. Reduced extension temperatures required for PCR amplification of extremely A+T-rich DNA. Nucleic Acids Res 24(8):1574-1575.

V. Single cell RT-PCR of *var* gene mRNA from *Plasmodium falciparum*

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Equipment

PCR machine with hot lid

Materials and reagents

GeneAmp RNA PCR Kit (Applied Biosystems)
RNase-free DNase, 10 U/μL (Stratagene)
DNase
RNase inhibitor
Opti-Prime PCR buffer 3 and buffer 4, mixed 1:1 (Stratagene)
*Taq*Start antibody (Clontech)
Taq DNA polymerase, 5000 U/μL (Pharmacia)

- Prepare PCR mix for *n* samples:

deoxyadenosine triphosphate:	2 μL × *n*
deoxycytidine triphosphate:	2 μL × *n*
deoxyguanosine triphosphate:	2 μL × *n*
deoxythymidine triphosphate:	2 μL × *n*
GeneAmp 10× PCR Buffer II:	2 μL × *n*
$MgCl_2$	4 μL × *n*
distilled water:	1 μL × *n*

- Aliquot the PCR mix into *n* thin-walled PCR tubes (15 μL/tube).
- Place a single infected erythrocyte into each tube and immediately freeze it on dry-ice (see PARASITES, section V:B).
- Release the DNA and RNA from the parasite by heating the tubes to 93 °C for 3 min in the PCR machine. Cool them on ice.
- Add 1 μL of DNase and 1 μL of RNase inhibitor to each tube. Degrade the DNA at 37 °C for 30 min in the PCR machine. Inactivate the DNase at 93 °C for 3 min.
- Add 1 μL of RNase inhibitor, 1 μL of random hexamers, and 1 μL of reverse transcriptase to each tube. Perform the reverse transcription at 42 °C for 30 min and inactivate the reverse transcriptase at 93 °C for 3 min.
- Prepare the *Taq*Start mix for *n* reactions:

Taq polymerase:	4.4 μL × *m*(where *m* = 4.8 × *n* / 25.4)
*Taq*Start antibody:	4.4 μL × *m*
*Taq*Start buffer:	17.6 μL × *m*

  Mix and incubate for at least 5 min at room temperature.

- Prepare the PCR master mix for *n* reactions:

Opti-Prime PCR buffer 3+4:	8 μL × *n*
Specific primer DBL1-1 (DBL1-3):	2 μL × *n*
Specific primer DBL1-2:	2 μL × *n*
*Taq*Start mix:	4.8 μL × *n*
distilled water:	63.2 μL × *n*

- Add 80 μL of PCR master mix to each tube. The tubes already contain 20 μL from the previous steps; the final volume in each tube should thus be 100 μL.
- Amplify the *var* sequences using the following PCR conditions:
  93° C for 20 s, 55 °C for 30 s, and 72 °C for 1 min.
  Run 50 cycles followed by a last extension step at 72 °C for 7 min.
- Run 20 μL of the PCR products on a 1.2% agarose gel with TBE buffer.

Primer sequences

DBL1-1:5′-GGW GCW TGY GCW CCW TWY MG-3′
DBL1-2:5′-ARR TAY TGW GGW ACR TAR TC-3′
DBL1-3:5′-GCA CGA AGT TTY GCA GA-3′

Reference

Chen Q, Fernandez V, Sundström A, Schlichtherle M, Datta S, Hagblom P, Wahlgren M. 1998. Developmental selection of *var* gene expression in *Plasmodium falciparum*. Nature 394(6691):392-395.

VI. Fluorescent *in situ* hybridization (FISH)$^{DNA\text{-}DNA}$ for *Plasmodium falciparum*

(Adapted from Ersfeld, K. & Gull, K, 1997)
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Reagents

thermal cycler with *in situ* adapter (Eppendorf)
waterbath at 37 °C
hybridization oven at 50°C
microscope

Materials and reagents

Saponin (Sigma)
RPMI 1640 (Gibco)
3 well microscope slides (Cell-Line) and coverslipes
In situ Frame for 25 µL (AbGene)
Hybridization solution (HS):
- 50% formamide (Roche Applied Science)
- 10% dextran sulfate (Sigma)
- 2× SSPE
- 250 µg/mL Herring sperm DNA (Sigma)

Probes: To prepare the probes, use the "Fluorescein High-prime kit" (Roche Applied Science) for Fluorescein signals and "Biotin high-prime kit" (Roche Applied Science). For detection of the biotinylated probes use avidin-conjugate with rhodamine (Roche Applied Science).

M solution (pH 7.5):
- 100 mM maleic acid (Sigma)
- 150 mM NaCl (Sigma)
- 1% blocking reagent (Roche Applied Science)
- 4% bovine serum albumin (BSA) (Sigma)

TNT solution (pH 7.5):
- 100 mM Tris–HCl
- 150 mM NaCl (Sigma)
- 0.5% Tween 20 (v/v) (Sigma)

20× SSC
10% paraformaldehyde (Electron Microscopy Sciences)
formamide (Roche Applied Science)
VECTASHIELD mounting medium with DAPI (Vector Laboratories)
nail polish

Procedure

Parasite fixation:

- Treat the parasites (10% parasitemia) with saponin to lyse the erythrocyte membrane (centrifuge 4000 rpm 5 min).
- Wash them twice in incomplete RPMI (centrifuge 6000 rpm 1 min).

- Resuspend the parasites in paraformaldehyde 4% (in PBS) for 10-15min, on ice.
- Wash once with cold PBS (centrifuge 6000 rpm 1 min).
- Resuspend in ~400 µL cold PBS (fixed parasites can be stored at 4 °C in this step for at least a week).
- Deposit a monolayer of parasites on each well of the microscope slide.
- Air-dry the slides for 30 min at room temperature.

In situ hybridization:

- Fix the plastic frame around the wells containing the parasites.
- Wash the slides once in PBS for 5 min at room temperature.
- If necessary, permeabilize using 0,1% Triton X-100 for 5 min and wash twice win PBS)
- Apply the HS solution with the labeled probe (denatured at 95 °C for 5 min) on each well.
- Cover the well with the *In situ* Frame coverslip.
- Denature the slides in a thermal cycler with *in situ* adapter for 30 min at 80 °C followed by hybridization at 37 °C overnight.

Washing:

- Remove the In situ Frame coverslip and the HS solution.
- Wash the slides in 2× SSC/50% Formamide for 30 min at 37 °C, followed by 1× SSC for 10 min at 50 °C, 2× SSC for 10 min at 50 °C, and 4× SSC for 10 min at 50 °C.
- Equilibrate the cells in M solution for 5 min at room temperature in a humid chamber protected from light.
- Remove the M solution and replace it with M solution plus Avidin–Rhodamine (1:10.000) for detection using biotin probes. Incubate the slides for 30 min at room temperature.
- Wash the slides three times in TNT solution 10 min each at room temperature, with agitation.
- Let air dry the slide and mount using Vectashield with DAPI.
- Analyze the slide by fluorescence microscopy.

Comments

This protocol allows a dramatic decrease of the background generated by the erythrocyte membranes due to the saponin lysis, and better preservation of nuclear architecture since the fixation step is done in suspension.

VII. Constructing *Plasmodium berghei* gene targeting vectors using recombineering

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1. Background

This protocol describes a restriction-ligation free method for engineering AT-rich *Plasmodium* DNA using the Red/ET recombination system of bacteriophage lambda (Zhang *et al*., 1998; Wang *et al*. 2006). We here use this method to generate vectors for gene knock-out and 3' tagging in *Plasmodium berghei* (Pfander *et al*. 2011). However, individual modules of the protocol can be combined and extended to generate more complex genetic modification and complementation vectors, even for large *P. berghei* genes. Recombineered vectors have two significant advantages over standard genetic modification constructs: 1) They are produced without PCR-based amplification of *Plasmodium* DNA (AT-rich and highly error-prone), and 2) they display an increased recombination frequency in *P. berghei* due to the augmented length of their homology arms of typically 6-10 kb.

This protocol uses clones from an arrayed large-insert library of (PbG) *P. berghei* genomic DNA, available from the Wellcome Trust Sanger Institute. The library has been end-sequenced and mapped, so individual library clones carrying a given gene of interest (GOI) can be identified and retrieved from frozen stocks. These clones are then converted into genetic modification vectors by using a combination of Red/ET recombineering and Gateway technology. Figure 1 outlines the steps involved. The PbG library currently contains >9000 clones, allowing for the design of knockout (ko) and c-terminal tagging vectors for >90% of all *P. berghei* predicted open reading frames. For PbG library clones and reagents contact plasmogem@sanger.ac.uk.

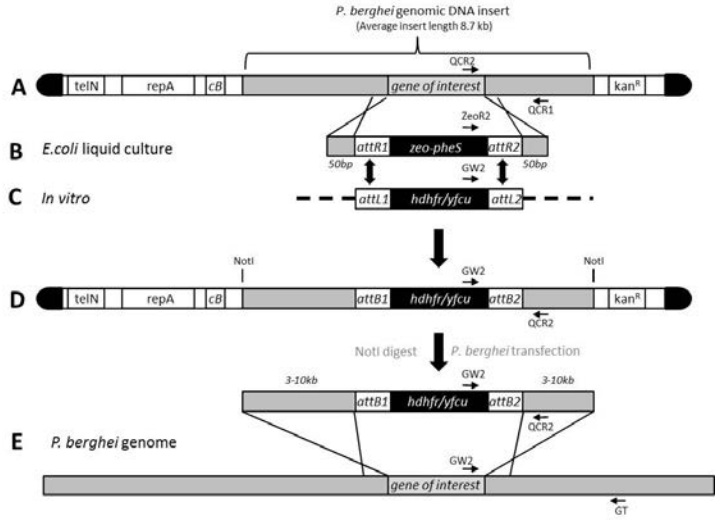

Figure 1. Schematic overview of the generation of *P. berghei* gene targeting vectors using recombineering. The strategy shown is that employed for targeted disruption. For c-terminal tagging the selection marker is inserted directly downstream of the gene of interest, replacing the

stop codon. **(A)** Schematic of the pJAZZ vector showing hairpin telomers (black), telomerase gene (*TelN*), replication factor and origin (*repA*), and kanamycin resistance gene (kan^R). The *P. berghei* genomic DNA insert carries the gene of interest (GOI). (**B**) The GOI is replaced by a bacterial selection (*zeo-pheS*) cassette by recombineering facilitated by 50 bp homology regions flanking the GOI, and present on a PCR product carrying the *zeo-pheS* cassette. (**C**) An *in vitro* Gateway (GW) reaction step exchanges the *zeo-pheS* marker for the *P. berghei hdhfr/yfcu* selection cassette, as mediated by the attR sites associated with the *zeo-pheS* marker, and attL sites flanking the *hdhfr/yfcu* cassette. (**D**) The completed gene targeting vector is prepared prior to *P. berghei* transfection by NotI digestion, which releases the modified *P. berghei* insert from the flanking vector sequences. **(E)** Following transfection, the gene targeting vector facilitates the modification the *P. berghei* genome by integration at the GOI through double homologous recombination.

2. Methodology summary:

A. Electroporation of pSC101gbaA plasmid and subsequent induction of recombineering competency proteins from this plasmid.

B. Electroporation of PCR product carrying the *zeo-pheS* bacterial selection cassette flanked by attR Gateway sites and 50 bp *P. berghei* homology regions. Recombination between homologous sequences is facilitated by the recombineering competency proteins, resulting in the replacement of the GOI with the *zeo-pheS* cassette, and generation of an intermediate vector (here denoted PbG zeo-pheS).

C. *In vitro* Gateway reaction using the pR6K attL1-3xHA-hdhfr-yfcu-attL2 (Pfander *et al*. 2011) as a donor plasmid. Exchange between attR sites on the PbG *zeo-pheS* intermediate vector and attL sites on the pR6K attL1-3xHA-hdhfr-yfcu-attL2 donor mediates the replacement of *zeo-pheS* with the *Plasmodium* positive negative selective marker *hdhfr/yfcu* (Braks *et al*. 2006).

3. Reagents and equipment

Electroporation events are performed using the Gene Pulser Xcell (Bio-Rad) or BTX ECM 630 (Harvard Scientific), and 1 mm electroporation cuvettes (Bio-Rad).

A. Selective media and agar

	Final conc.	Stock conc.	Solvent
Kanamycin	30 µg/ml	10 mg/ml	dH_2O
Tetracycline	5 µg/ml	5 mg/ml	ethanol
Zeocin (Invitrogen)	50 µg/ml	10 mg/ml	dH_2O

Terrific broth (TB): For 1 L TB use 11.8 g Bacto-tryptone, 23.6 g yeast extract, 9.4 g dipotassium hydrogen phosphate (anhydrous) and 2.2 g potassium dihydrogen phosphate (anhydrous), supplemented with 0.4% glycerol after autoclaving.

YEG-Cl kanamycin plates: Per 1 L dH_2O, add 5 g Yeast Extract, 5 g NaCl, 2 g 4-chloro-DL-phenylalanine and 15 g agar and autoclave. Allow to cool to < 55°C, add 0.4 % (v/v) sterile glucose and kanamycin.

Zeocin plates: Per 1 L dH_2O, add 5 g yeast extract, 5 g NaCl and 15 g agar and autoclave. Allow to cool to < 55°C and add zeocin (Source Bioscience).

B. Miscellaneous reagents

10% L-arabinose, sterile filtered.
Gateway LR Clonase enzyme (Invitrogen), (kit contains buffer and Proteinase K).
BigEasy-TSA Electrocompetent Cells (SOLOS), (Lucigen).
100µM pore filters for DNA dialysis (Millipore).
Sterile HPLC-grade H_2O is used throughout the protocol.
MilliQ ultrapure H_2O used for media.
Qiaprep Spin Mini Prep Kit (Qiagen).
PCR reagents: Proof-reading polymerase (e.g. Advantage 2 polymerase, Clontech).
2xGoTaq Green master mix (Promega), screening polymerase.
DpnI restriction enzyme.

C. Plasmids

PbG clones: PbG is a *P. berghei* ANKA clone 15cy1 genomic DNA library. The library was constructed using the pJAZZ-OK blunt low copy (five per cell) vector from Lucigen (Ravin *et al.* 2003, Godinska *et al*. 2010). These clones and their derivatives can only be propagated in TSA *E.coli* (ampicillin^R, Lucigen). This vector confers kanamycin resistance.

pSC101gbdA: This plasmid encodes recombination and proofreading activities from lambda phage and recA. This vector converts any *E. coli* strain into a competent strain for Red/ET recombination (Wang *et al.* 2006) and confers tetracycline resistance. The pSC101 origin of replication restricts replication of this plasmid to 30°C. In contrast, 37°C is non-permissive. L-arabinose induction and temperature switching together tightly regulate Red/ET protein expression.

pR6K attR1-zeo-pheS-attR2: The template for the PCR reaction amplifying the *zeo-pheS* selection marker with flanking attR1 and attR2 sites for Gateway recombination. This vector confers tetracycline resistance and can only be replicated in *pir*$^+$ *E.coli*.

pR6K attL1-3xHA-hdhfr-yfcu-attL2: The Gateway donor plasmid that facilitates the replacement of the *zeo-pheS* marker with the *hdhfr-yfcu* casette. This plasmid is available with different flavour tags for c-terminal addition of GFP or 3xHA tags; other tagging vectors are in development. This vector confers tetracycline resistance and can only be replicated in *pir*$^+$*E. coli*.

D. Primers

Primer sequences are available from http://plasmogem.sanger.ac.uk.

Primers for amplification of the *zeo-pheS* selection cassette: Primers contain 50 bp of sequence specific for GOI, followed by 20 bp annealing to the selection cassette.

Primer R1: 50 bp homology region specific for GOI + 5'-aaggcgcataacgataccac-3'
Primer R2: 50 bp homology region specific for GOI (Reverse complement) + 5'-ccgcctactgcgactataga-3'

Primers for amplification of the *wt* GOI allele: Primers are designed to span the insertion site for the *zeo-pheS* alternatively, *hdhfr-yfcu* cassette. Prior to starting, the primer combination below can be used to confirm the presence of the GOI in the PbG clone used as starting material.

QCR1: 20 bp primer annealing within the modified or deleted GOI
QCR2: 20 bp primer annealing outside modified or deleted GOI

Primers for assessment of successful integration of selection cassettes into PbG clone, when used in combination with the gene specific QCR2 primer:

zeo-pheS cassette: ZeoR2 5'-tcattcttcgaaaacgatct-3'
hdhfr-yfcu cassette: GW2 5'-ctttggtgacagatactac-3'

Protocol

***Day 0**. Start PbG clone culture*

0.1 Inoculate PbG clone from glycerol stock into 4.0 ml TB-kanamycin.
0.2 Grow overnight at 37°C, shaking at ~250 rpm.

***Day 1**. Transformation of recombinase plasmid pSC101gbdA*

Before start, chill H_2O and electroporation cuvettes on ice and cool centrifuge to 4°C.
1.1 Dilute overnight cultures to an OD_{600} of 0.05, in 4 ml TB-kanamycin.
1.2 Resume shaking at **37°C** until OD_{600} reaches 0.6-0.8 (check OD_{600} after 2 hours).
1.3 During incubation:
  a. Set up *zeo-pheS* cassette PCR with gene specific homology arms (1.12).
  b. Dilute pSC101gbdA plasmid to 10 ng of plasmid in 50 µl H_2O. Keep on ice.
1.4 When at an OD_{600} of 0.6-0.8, place the tube with the culture on ice for 15 min. Keep cells cold from this point onwards.
1.5 Transfer 1.4 ml of culture to 1.5 ml centrifuge tube. Discard the rest of the culture.
1.6 Spin for 3 min at 5,000 g at 4°C.
1.7 Make cells electrocompetent by sequential washes in ice cold HPLC-grade H_2O:
  1.7.1 Carefully aspirate supernatant
  1.7.2 Wash the cell pellet in 1 ml ice cold H_2O.
  1.7.3 Spin 3 min at 5,000 g in a cold microfuge.
  1.7.4 Repeat 2 more washes (step 1.7.1-1.7.3) with 1 ml ice cold H_2O.

The cell pellet becomes looser with each wash. Make sure cells are drained well after final wash.
1.8 Keeping the tube on ice, re-suspend cell pellet in 50µl of diluted pSC101-gbdA plasmid, and transfer to a chilled 1 mm gap-width electroporation cuvette.
1.9 Electroporate bacteria:
  1.9.1 Settings: BTXECM 630 electroporator (1800 V, 25 µF, 200 Ω), or Bio-Rad Gene Pulser Xcell (1800 V, 10 µF, 600 Ω).
  1.9.2 Immediately add 950 µl of TB, and transfer cells to a 14 ml culture tube.
  1.9.3 Allow cells to recover at **30°C**, shaking at 225 rpm for 70 min. Total volume at this stage is 1 ml.
  DO NOT CULTURE AT 37°C AT THIS STAGE.
1.10 Add 3 ml TB supplemented with kanamycin and tetracycline (final concentration; 30 µg/ ml kan and 5 µg/ml tet), incubate o/n at **30°C** shaking.
  DO NOT CULTURE AT 37°C AT THIS STAGE.

1.11 PCR amplification of *zeo-pheS* cassette with homology arm extensions for recombineering:

1.11.1 PCR reaction

H_2O	15.5µl
pR6K attR1-zeo-pheS-attR2 plasmid template(12 ng/µl)	1.0µl
10x PCR buffer	2.5µl
Primers R1 and R2 (2 µM), each	2.5µl
dNTPs (10 mM each)	0.5µl
AdvantageTaq2 (Clontech), or other proof-reading Taq polymerase	0.5µl

95°C 5' // 95°C 30'' / 58°C 30'' / 72°C 1'30'' (x30) // 72°C 10' // 4°C hold

1.11.2 Visualise 2.5 µl of PCR product by gel electrophoresis. Expected size is 2.0 kb.

1.11.3 Digest the rest of the PCR reaction with 1 µl of DpnI (stock 20 U/µl) at 37°C for 1 h. This is done to eliminate template plasmid before transformation.

1.11.4 Dialyse PCR product against HPLC-grade H_2O (0.1 µM pore filters, Millipore) for 1h.

1.11.5 Transfer dialysed product from the filter to an Eppendorf tube, quantify DNA by spectrophotometry and store at -20°C.

Day 2. *Transformation of zeo-pheS bacterial selection cassette (recombineering)*

Before start, chill H_2O and electroporation cuvettes on ice and cool bench top centrifuge to 4°C.

2.1 Dilute overnight cultures to an OD_{600} of 0.05, in 4 ml TB with kanamycin and tetracycline.

2.2 Resume shaking at **30°C** until OD_{600} reaches 0.3-0.4 (check OD_{600} after 2 h). DO NOT CULTURE AT 37°C AT THIS STAGE.

2.3 During incubation:

2.3.1 Prepare attR1-zeo-pheS-attR2 PCR product for transformation by diluting 250ng – 1µg of purified PCR product in 50 µl of cold ddH_2O.

2.4 At OD_{600}=0.3-0.4, add 80 µl 10% L-arabinose (0.2% final concentration) and incubate:

2.4.1 In water bath for 5 min at **37°C.**

2.4.2 In shaking incubator for 35 min at **37°C.**

2.5 Chill tube on ice for 15 min. Keep cells cold from this point onwards.

2.6 Transfer 1.4 ml of culture to 1.5 ml centrifuge tube. Discard the rest of the culture.

2.7 Spin for 3 min at 5,000 g at 4°C.

2.8 Make cells electrocompetent by sequential washes in ice cold HPLC-grade H_2O as outlined in step 1.7.

2.9 Keeping the cells on ice, re-suspend cells in 50 µl of diluted attR1-zeo-pheS-attR2 PCR product, and transfer to a chilled 1 mm gap-width electroporation cuvette.

2.10 Electroporate cells as outlined in step 1.9.

2.11 Allow cells to recover at **37°C**, shaking for 70 min. Total volume at this stage is 1 ml.

2.12 Add 3 ml of TB supplemented with zeocin (final concentration 50 µg/ml) and incubate o/n at **37°C** shaking.

Important note. One of the strengths of recombineering is its high efficiency, which allows selection to take place through serial liquid culture. However, transformations can be plated out at this stage to allow for screening of single colonies. This ensures that

only successfully recombineered clones are taken forward to the next step. This approach may be favoured when first establishing the protocol in your lab, or when a particular construct proves difficult to generate.

Single colony screening is thus not routinely necessary, but if it is the preferred method, after step 2.11; first remove 100 µl culture and keep to one side. Spin remaining culture volume for 2 minutes at 6000 rpm, remove ~800 µl of the supernatant and then re-suspend cells in the remaining 100µl. Spread the two dilutions of cells onto pre-warmed zeocin agar plates, incubate o/n at 37°C, then screen individual colonies by PCR (as outlined in step 3.3) for successful insertion of the *zeo-pheS* cassette. Single *zeo-pheS* positive colonies can then be grown in 2 ml TB-zeocin and the protocol resumed at step 3.1.

Day 3. *Gateway mediated replacement of zeo-pheS selection cassette with hdhfr-yfcu marker*

3.1 Use the Qiaprep Spin Mini Prep Kit (Qiagen) according to manufacturer's instructions to isolate the intermediate vector containing *zeo-pheS.* Use 2 ml culture volume and double P1, P2, N3 buffer volumes to account for growth in rich TB medium. Elute in 50 µl of TE pH 8.0.

3.2 Quantify DNA and visualise PbG *zeo-pheS* intermediate vector by gel electrophoresis. A single, or two very closely migrating, band(s) of >12kb should be present.

3.3 Verify the recombineering reaction by PCR:

3.3.1 PCR reaction

H_2O	6.5 µl
Template (plasmid DNA)	1.0 µl
2xGoTaq Green master mix	12.5 µl
Primer QC2 (Gene specific), (2 µM)	2.5 µl
Primer ZeoR2 (Generic), (2 µM)	2.5 µl

95°C 5' // 95°C 30'' / 50°C 30'' / 68°C 1' (x30) // 68°C 10' // 4°C hold

3.3.2 Visualise 10 µl of PCR product by gel electrophoresis. The resulting PCR product should migrate as a single band of ~400-800 bp.

3.4 Set up the Gateway LR Clonase reaction in a thin-walled PCR tube:

PbG zeo-pheS intermediate vector (~30 ng/µl)	10.00 µl
pR6K attL1-3xHA-hdhfr/yfcu-attL2 (100 ng/µl)	1.00 µl
LR clonase buffer 5X	4.00 µl
LR clonase enzyme mix	2.00 µl
TE	3.00 µl

3.5 Incubate the Gateway mixture o/n at 25°C in a PCR machine using heated lid function.

Day 4. *Transformation of Gateway product*

4.1 Add 0.5 µl of proteinase K to inactivate Gateway reaction and incubate at 37°C for 10 min.

4.2 Dialyse the Gateway product as described in step 1.12.4.

4.3 Transform 5-10 µl of dialysed Gateway product into 50 µl BigEasy-TSA electrocompetent cells, as described in step 1.9.

4.4 Allow cells to recover at **37°C**, shaking for 70 min. The volume at this stage is 1 ml.

4.5 Remove 100 µl culture, and keep to one side.

4.6 Transfer remaining culture volume to 1.5 ml Eppendorf tubes.
4.7 Spin remaining culture volume for 2 minutes at 6000 rpm, remove ~800 µl of the supernatant, and re-suspend cells in the remaining 100 µl culture medium.
4.8 Spread the two dilutions of cells onto pre-warmed YEG-Cl kanamycin agar plates.
4.9 Incubate plates overnight at **37°C**.

Day 5. *PCR based screening for Gateway hdhfr-yfcu positive PbG clones*
5.1 Pick >4 colonies and perform colony PCR to verify the Gateway reaction:
5.1.1 Pick single colonies and inoculate 4 ml TB-kanamycin and 10 µl H_2O:
5.1.1.1 Grow inoculated selective medium o/n at 37°C for glycerol stocks.
5.1.1.2 Boil 10 µl colony lysate for 10 min at 95°C.
5.1.2 PCR Reaction

H_2O	5.0 µl
Template(colony lysate)	2.5 µl
2xGoTaq Green master mix	12.5µl
Primer QC2 (gene specific), (2µM)	2.5 µl
Primer GW2 (generic), (2µM)	2.5 µl

95°C 5' // 95°C 30'' / 50°C 30'' / 68°C 1' (x30) // 68°C 10' // 4°C hold

Important note. Verified clones can be prepared for *P. berghei* transfection by growing medium or large scale cultures in TB supplemented with kanamycin. Qiagen Midi or Maxi prep kit can then be used to purify the DNA (using double buffer volumes as described in step 3.1). Prior to transfection, the entire *P. berghei* genomic DNA fragment, which contains the modified GOI and acts as the targeting vector, is released by NotI digestion. *P. berghei* transfections are performed using 1-5 µg NotI digested DNA, purified by standard ethanol precipitation. There is no need to purify the insert away from the pJazz flanking arms that are also released by NotI digestion.

References

- Braks JA, Franke-Fayard B, Kroeze H, Janse CJ, Waters AP (2006) Development and application of a positive-negative selectable marker system for use in reverse genetics in *Plasmodium*. Nucleic Acids Res **14**, 34-35.
- Godiska, R.*et al.* (2010) Linear plasmid vector for cloning of repetitive or unstable sequences in *Escherichia coli*. *NAR***38**, e88.
- Pfander, C., Anar, B., Schwach, F., Otto, T.D., Brochet, M., Volkmann, K., Quail, M.A., Pain, A., Rosen, B., Skarnes, W.C., Rayner, J.C., and Billker, O. A scalable pipeline for highly effective genetic modification of a malaria parasite. Nature Methods Volume **8**, 1078–1082.
- Ravin, N. V., Kuprianov, V. V., Gilcrease, E. B. &Casjens, S. R. (2003) Bidirectional replication from an internal ori site of the linear N15 plasmid prophage. *NAR***31**, 6552-6560.
- Wang, J.*et al.* (2006) An improved recombineering approach by adding RecA to lambda Red recombination. *Mol. Biotech.***32**, 43-53.
- Zhang, Y., Buchholz, F., Muyrers, J. P. & Stewart, A. F. (1998) A new logic for DNA engineering using recombination in *Escherichia coli*. *Nature Genetics* **20**, 123-128.

VIII. Separation and mapping of chromosomes of malaria parasites using pulsed-field gel electrophoresis (PFGE)

VII:A. PFGE Protocol 1

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Equipment

Pulsaphor/Gene Navigator PFGE apparatus (Pharmacia) or CHEF-DR system Bio-Rad)
centrifuge
water bath
15-mL Falcon tubes (BD Labware)

Materials and reagents

SeaKem GTG agarose (Cambrex) for agarose gels at a concentration of >0.6%
chromosomal grade agarose (Bio-Rad) for agarose gels ≤0.6%
low-melting point agarose (Cambrex) for embedding parasites in agarose blocks and preparative isolation of chromosomes
plastic gel molds (2 × 5 × 10 mm, 100-μL volume; Pharmacia)
nylon membrane: Hybond-N+ (Amersham) or Biotrans(+) (ICN Biomedicals)
These membranes allow the rapid alkaline transfer of DNA and their physical characteristics make them especially useful for multiple hybridization cycles (usually 5 to 10 cycles).
erythrocyte lysis buffer: 0.15% saponin (Sigma) in phosphate-buffered saline (PBS).
ethidium bromide in water: 10 mg/mL
CAUTION: This chemical is toxic and mutagenic. Wear protective clothing and use only in a chemical fume hood.

alkaline transfer buffer: 0.4 M NaOH
20× SSC:
3 M sodium chloride
0.3 M sodium citrate
TE buffer:
10 mM Tris-HCl
1 mM EDTA (pH 8.0)
1 mM phenylmethylsulphonyl fluoride (PMSF) in TE

molecular weight size standards:
5 kb ladder (4.9 to 120 kb)
ladder (0.05 to 1 Mb)
Saccharomyces cerevisiae (0.2 to 2.2 Mb)
Hansenula wingei (1 to 3.1 Mb) (Bio-Rad)

cell lysis buffer (CLB):
0.5 M EDTA (pH 8.0)
10 mM Tris–HCl
1% sodium lauryl sarcosinate (Sigma)

Store at room temperature. Before cell lysis, add 2 mg/mL of proteinase K from a stock solution.

prehybridization and hybridization buffer:
0.5 M $NaPO_4$ (pH 7.2)
7% SDS
1% BSA

hybridization buffer:
7% SDS
0.5 M EDTA (pH 8.0)
1% BSA

saturated 2-butanol:

Mix *2*-butanol with 1 M NaCl in TE (4:1 v/v). Add 9 volumes of saturated *2*-butanol and 1 volume of the aqueous phase to the gel blocks. This is important to avoid gel shrinking!

10× TBE buffer:
108 g Tris-base
54 g boric acid
8.35 g disodium-EDTA per liter (pH ~8.5)

Preparation of DNA for PFGE

The ability to preserve the intact size of large DNA molecules is critical for the success of PFGE. To overcome the problem of DNA shearing, intact cells are embedded in low-melting agarose blocks. Cells are lysed and proteins are removed by proteinase K treatment. This procedure yields DNA that is both intact and susceptible to restriction enzyme digestion. The agarose block can be loaded directly into the well of a pulsed-field gel.

The host cells of the intracellular blood stage form of *Plasmodium falciparum* need to be lysed by saponin before the parasite cells are embedded into agarose blocks.

- Estimate the volume of the erythrocyte pellet and add 1.5 volumes of 0.15% saponin in PBS at room temperature.
- Resuspend the pellet and incubate it for 3 to 5 min. The liquid will clarify as the red blood cells lyse.
- Add 5 volumes of cold PBS and centrifuge the pellet at 5,000 rpm for 10 min.
- Resuspend the dark pellet which contains the parasite material in 2 mL of PBS.
- Pellet the cells by centrifugation at 3,000 rpm for 10 min.
- Resuspend the pellet at a concentration of approximately 5×10^8 parasites/mL of PBS.
- Equilibrate the cell suspension at 37 °C and add an equal volume of 1.6% melted low-melting point agarose (37 °C).
- Mix the cell suspension gently and dispense it immediately into plastic gel molds.
- Allow the gel to solidify at 4 °C for about 20 min.

- Place up to 20 solidified blocks in 10 mL of cell lysis buffer (CLB) and incubate them for 24 h at 42 °C.
- Replace the CLB with an equal volume of fresh CLB.
- Incubate the blocks for an additional 24 h.
- The blocks can be stored indefinitely in TE buffer at 4 °C.

Electrophoresis conditions

Optimal separation of the different chromosome size classes usually requires the use of a range of different PFGE conditions. Table 1 shows the running conditions of large DNA fragments of various sizes using the CHEF (clamped homogeneous electric field) system. These PFGE methods have been used in studies of *Plasmodium falciparum* and *Trypanosoma cruzi*, but also apply to other parasitic protozoa. The described electrophoresis conditions have been developed for the Pulsaphor/Gene Navigator PFGE apparatus (Pharmacia) based on the CHEF design. Similar results have been obtained with the CHEF-DR system (Bio-Rad).

Table 1. Electrophoresis conditions for separation of DNA fragments by PFGE (CHEF system)

Efficient separation	Pulse time (ramping)	Voltage	Run time	Agarose concentration	Buffer
5–100 kb	0.5 s –1.0 s	400 V	7 h	1.3%	0.2× TBE
50–2000 kb	50 s – 90 s	200 V	22 h	1.0%	0.5× TBE
500–4000 kb	90 s – 300 s for 24 h followed by 300 s – 720 s for 24 h	95 V 85 V	48 h	0.7–0.8%	0.5× TBE
2000–6000 kb	120 s – 720 s	85 V	36 h	0.4–0.6% high gel strength agarose	0.5× TBE

- The agarose concentration and type of agarose used depends on the DNA size range to be separated. For example, pulsed field separations larger than 2 megabases are improved by using high gel-strength agarose (chromosomal grade agarose, Bio-Rad) allowing preparations of very low-percentage agarose gels (0.4 to 0.6%), which give significantly better separation of large chromosomes in the range of 2 to 5 megabases..
- For a 15 cm × 15 cm × 0.5 cm 1% agarose gel, dissolve 1.1 g of SeaKem GTG agarose in 110 mL of 0.5× TBE buffer by heating it in a microwave oven. Make sure that the volume has not changed. If it has, adjust again to 110 mL.
- Cool the agarose in a 50 °C water bath and pour it into the casting stand.
- After the gel is solidified, fill the wells with 0.5× TBE buffer and insert the blocks containing parasite DNA. About 2 to 5 mm of a block (1×10^7 to 2.5×10^7 parasites) generally contains enough material to visualize the chromosomes after ethidium bromide staining. (This number may also work for other parasite

species.) However, for best PFGE results it may be necessary to determine the optimal number of cells/block.
- Seal the blocks into the well with 1% low-melting point agarose.
- Fill the electrophoresis chamber with 2.2 liters of TBE buffer. Do not overfill; the buffer should just cover the top of the agarose gel. Keep the temperature of the running buffer (18 °C) constant during the run.

Staining of chromosomes
- After completing the PFGE run, the separated chromosomes can be visualized by staining the agarose gel for 15 min in ethidium bromide (1 μg/mL in water). Destain the gel by two washes in 0.5× TBE for 1 h with gentle agitation, and photograph it (do not forget the ruler) using a shortwave UV light (254 nm). **ATTENTION!** If chromosomes are intended for use in subsequent restriction mapping studies, longwave UV light (360 nm) must to be used to avoid nicking the DNA.

Southern hybridization
- Transfer the DNA from the agarose gel to a nylon membrane such as Hybond-N or Biotrans. In order to ensure efficient transfer of large DNA fragments, nick the chromosomal DNA by exposing the stained agarose gel for 5 min on a UV-light table (254 nm).
- The alkaline transfer procedure using 0.4 N NaOH as transfer buffer works fine for chromosomal DNA blots. We routinely set up the capillary transfer (standard molecular biology procedure) for at least 24 h. Make sure that the weight on top of the absorbent paper stack does not exceed 1 kg. There is no need to fix DNA after alkali blotting.
- For ^{32}P-labelled DNA probes, the random hexamer priming method in combination with the following hybridization buffer usually works well to give a good signal-to-background ratio with nylon membranes: 7% SDS, 0.5 M EDTA (pH 8.0), 1% BSA. Single-copy genes of *P. falciparum* are generally detected after 6 h to overnight exposure on X-ray film.
- Remove probes by standard protocols or as recommended by the manufacturer. Stripped blots can be stored for prolonged periods at room temperature.

Restriction enzyme digestion of embedded chromosomes

Generally, restriction enzymes diffuse into agarose blocks and are thus suited for chromosome mapping studies. The most useful restriction endonucleases are those with 8-base recognition sites which cut only a few times in chromosome-sized DNA fragments. However, in genomes which are biased in their AT content, such as *P. falciparum*, certain enzymes which recognize GC-rich 6-base pair sequences (*Sma*1, *Bgl*1) can also be used as “rare” cutters in these AT-rich genomes.
- For restriction enzyme digestion of agarose-embedded total parasite DNA, remove the lysis buffer by extensive washes in TE buffer (2 to 3 washes in 20 mL of TE).
- Inactivate any remaining proteinase K by treating the blocks with 1 mM phenylmethylsulphonyl fluoride (PMSF) in TE (10 mL for 20 blocks) for 2 h at room temperature, followed by three washes in 10 mL of TE for 30 min each.

Individual chromosomes or DNA fragments that have been cut out of a stained agarose gel after a PFGE run must be treated with 2-butanol to extract any remaining ethidium bromide prior to restriction enzyme digestion.

- Add 9 volumes of 2-butanol saturated in 1 M NaCl/TE and 1 volume of the aqueous phase to the gel block (5:1, v/v).
- Agitate the mixture at room temperature for 30 min.
- Repeat the extraction twice and wash the block five times in 10 mL of TE buffer for 30 min each.
- Store the blocks at 4 °C.
- Equilibrate the blocks with 5 volumes of restriction buffer containing 100 μg/mL of nuclease-free BSA for 30 to 60 min at room temperature. Remove the buffer and add 2 volumes of fresh buffer and restriction endonuclease (approximately 100 to 200 U/mL). Incubate the blocks at the recommended temperature for 4 h or overnight.
- Partial digestion of chromosomal DNA is useful for mapping studies. In this case, set up separate digestions of serial dilutions of the restriction enzyme for 2 h each (0.01 U/mL, 0.1 U/mL, 1 U/mL, 10 U/mL, and 100 U/mL. Stop the reactions by adding EDTA to a final concentration of 50 mM.
- Depending on the expected fragment size, restricted DNA can be separated by PFGE according to the run conditions described in Table 1.

Two dimensional PFGE

Most chromosomes in *P. falciparum*, as in other protozoan parasites, are heterogeneous in size and can be separated from each other by PFGE. Two-dimensional (2D) PFGE studies of a karyotype can give useful information concerning genetic markers located on several chromosomes. Multigene families are often observed in pathogenic protozoa.

- Separate chromosomes in the first dimension and stain them with ethidium bromide as described above.
- Photograph the gel using longwave UV light (360 nm).
- Use a razor blade to excise strips of gel (~2 mm) that contain all chromosomes.
- Extract ethidium bromide using 2-butanol in a 15-mL Falcon tube (as described above).
- Perform restriction enzyme digestions as described above.
- Embed the slice of gel containing the digested chromosomes and size markers (λ ladder or 5 kb ladder) into a precut slot in the top of a 1.3% agarose gel and seal it with 1% low-melting point agarose.
- Carry out standard PFGE separation using the run conditions described in Table 1. For Southern hybridization, continue as described above.

References

Hernandez-Rivas R, Scherf A. 1997. Separation and mapping of chromosomes of parasitic protozoa. Mem Inst Oswaldo Cruz 92(6):815-819.

Hinterberg K, Scherf A. 1994. PFGE: improved conditions for rapid and high-resolution separation of *Plasmodium falciparum* chromosomes. Parasitol Today 10(6):225.

VII:B. PFGE Protocol 2

by ***Mats Wahlgren***
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Equipment

centrifuge
incubator
Plug mold (Bio-Rad)
PosiBlot 30-30 pressure blotter (Stratagene)
Stratalinker UV crosslinker (Stratagene, model 2400)
Falcon tubes (BD Labware)

Materials and reagents

10% saponin in PBS
10 mM EDTA (pH 7.6)
PBS
low-melt agarose
0.5 M EDTA, 1% *N*-lauryl sodium sarcosinate (sarkosyl), proteinase K
chromosomal grade agarose (Bio-Rad)
ethidium bromide

CAUTION: This chemical is toxic and mutagenic. Wear protective clothing and use only in a chemical fume hood.

0.25 M HCl
denaturation buffer:
0.5 M NaOH
1.5 M NaCl
neutralization buffer:
1 M Tris
1.5 M NaCl
10× SSC
nylon membrane Hybond-N (Amersham)

TSE:
10 mM Tris–HCl (pH 8.0)
100 mM NaCl
1 mM EDTA

Sample preparation

- Calculate the parasitemia of the culture to be used.
- Add 1/100 volume of 10% saponin to the culture. Incubate the culture for 5 min at room temperature.
- Pellet the free parasites by centrifuging them at ~1,000 rpm for 5 min (i.e., a “gentle spin”).
- Dissolve the pellet in PBS and spin it again. When dissolving the pellet, use a broad tip.
- Wash the pellet two more times with PBS.

- After the last centrifugation, dissolve the pellet in TSE to get a concentration of 8 $\times$ 10^8 parasites/mL.
- Mix the parasites with an equal volume of 1.6% low-melt agarose to get a final concentration of 4 $\times$ 10^8 parasites/mL. The agarose should be between 37 and 42 °C.
- Dispense the mix into plug molds and let the agarose solidify, first at room temperature and then at 4 °C for 20 to 30 min.
- Put the DNA/agarose plugs in 0.5 M EDTA, 1% *N*-lauryl sodium sarcosinate, 2 mg/mL of proteinase K; 10 mL of solution will be enough for 20 DNA plugs in a Falcon tube.
- Incubate the plugs at 37 °C for 48 h.
- Store the DNA plugs at 4 °C in the same solution as above (or possibly in TE).

Electrophoresis conditions

See also the section on electrophoresis conditions in MOLECULAR BIOLOGY, section VII:A, for details.

Separation		Pulse time (ramping)	Voltage	Run time	Included angle	Buffer/ agarose *
Quick separation 500–2500 kb	Blk 1	5–12 min	2.0 V/cm	30 h	53°	1× TAE/ 0.7%
	Blk 2	3–5 min	4.0 V/cm	17.5 h	53°	
Good separation 500–1800 kb	Blk 1	6–8 min	2.5 V/cm	80 h	60°	0.5× TBE/ 1%
	Blk 2	4–6 min	2.5 V/cm	64 h	60°	
	Blk 3	2–4 min	2.5 V/cm	20 h	60°	

***** The agarose used is chromosomal grade agarose (Bio-Rad). Buffer temperature is 14 °C for quick separation and 10 °C for good separation.

Southern blot

- Stain the gel for 30 min in 1 μg/mL of ethidium bromide. Destain the gel for 10 min in distilled water. Look at the gel under UV light and take a photograph. Destain the gel for another hour in distilled water.
- Prepare the gel for Southern transfer by depurinating it in 0.25 M HCl for 30 min.
- Rinse the gel in distilled water and denature it in denaturation buffer for 30 min.
- Rinse the gel in distilled water and neutralize it in neutralization buffer for 30 min.
- Rinse the gel in 10× SSC. Wet the nylon membrane in distilled water and let it soak for 5 min in 10× SSC.
- Transfer the chromosomes from the gel to the nylon membrane in a PosiBlot 30-30 pressure blotter. Assemble the pressure blotter according to the manual and use 10× SSC as the transfer buffer. One hour at 75 to 80 mm Hg is enough to transfer the chromosomes to the membrane.
- Quickly rinse the filter in 2× SSC to get rid of any gel pieces sticking to the membrane. Put the membrane on a Whatman paper to dry off a little.

- While the membrane is still moist, cross-link the DNA to the membrane by exposing it to UV light in a crosslinker. Use setting “auto cross-link”.

References

Chen Q, Fernandez V, Sundström A, Schlichtherle M, Datta S, Hagblom P, Wahlgren M. 1998. Developmental selection of *var* gene expression in *Plasmodium falciparum*. Nature 394(6691):392-395.

Dolan S, Adam R, Wellems T. 1993. Chromosome mapping methods for parasitic protozoa. *In:* Hyde JE, ed. Methods in molecular biology. Vol 21. Totowa, NJ, USA: Humana Press; p 319-332.

VII:C. PFGE Protocol 3

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see: Transfection IV, B, page 374

IX. Production of recombinant *Plasmodium* proteins

IX: A. Recombinant expression of *Plasmodium falciparum* var-gene domains

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Equipment

bacterial culture flask
bacterial culture incubator
sonicator/French press
centrifuge (4 °C)
spectrophotometer

Materials and reagents

pET vector (Novagen) or any vector with T7 promoter
Shuffle T7 express competent *E. coli* or Shuffle T7express lysY competent *E. coli* (New England Biolabs)
LB plates (ampicillin 100 µg/mL)
PBS (pH 7.2)
Terrific Broth (TB - pH 7.2)/amp medium:
- 12 g/L casein enzymic hydrolysate
- 24 g/L yeast extract
- 2.2 g/L monopotassium phosphate
- 9.4 g/L dipotassium phosphate
- 4ml glycerol
- ampicillin 100 µL/mL

Lysis Buffer:
- 100 mM Hepes
- 250mM NaCl
- 10% Glycerol
- 10% Sucrose

Complete Protease Inhibitor Cocktail EDTA-free (Roche Applied Science)
lysozyme (Roche Applied Science)
Imidazole
Ni-NTA Agarose (Qiagen) or Talon Affinity Resin (Clontech)

Procedure

- Clone the sequence of *var*-domains into the pET vector in the correct reading frame.
- Transform *Shuffle T7 express competent* E. coli *or Shuffle T7express lysY competent* E. coli cells and select the positive transformed bacteria with ampicillin in LB (100 μg/mL) using manufacturer's conditions. The latter strain can be used when protein results toxic for the bacteria.
- Pick several colonies and culture them separately overnight in 5 mL of LB medium with 100 μg/mL of ampicillin at 37 °C.
- Inoculate the overnight culture in prewarmed (room-temperature) TB/amp medium in a ratio of 1:200. Use 20 mL of medium for the initial expression test,

and 1 to 10 liters for large-scale expression. Continue shaking the culture at 30 °C until the OD_{600} of the culture is 0.6.

- Check the induction system for your vector. If you are using the pET system and thus the *lac*-promoter, add IPTG to 0.4 mM (using a 1 M stock) and induce the expression at a 16°C overnight (at least 16-20hours).
- Sediment the bacteria by centrifugation at 1,500 × *g* for 20 min at 4 °C if using large-volume centrifuge flasks. For small volumes, sediment for 10 min.
- Resuspend the bacteria with Lysis Buffer (5ml/g of pellet):
  100 mM Hepes, 250mM NaCl, 10% Glycerol, 10% Sucrose
  Protease Inhibitor cocktail to 1 mM
  lysozyme to 1 mg/mL
- Shake at 22°C for 1 hour
- Mix completely and sonicate the bacteria on ice for five minutes with 10 s pulses, or use a French press to release the fusion protein from the bacteria. You may have to pass the bacterial solution through the press twice to release protein.
- Centrifuge bacterial lysis at 50,000 × *g* for 15 min at 4 °C.
- Remove and save the supernatant (note: the supernatant can be stored at -80°C until purification).
- If using a his-vector, prepare Ni-NTA Agarose (or Talon) according to manufacturer's instructions and follow the protocol below. Otherwise, refer to the manufacturer's recommendations for the expression system you have chosen.
- Pack a 5ml column using the bead slurry.
- Wash with PBS+10mM imidazole using at least 10 times the column volume (CV)
- Add imidazole (to 10mM final) to the bacterial supernatant and apply it to the column.
- Wash with PBS+10mM imidazole using 10x CV or until A_{280} reaches zero.
- Elute the his-tagged protein using PBS+250mM imidazole. Monitor the elution by measuring the A_{280} and collect 1ml fractions until A_{280} reaches zero.
- Aliquot, then store the fusion protein at −70 °C.

Reference

Angeletti D, Albrecht L, Wahlgren M, Moll K. 2013. Analysis of antibody induction upon immunization with distinct NTS-DBL1α-domains of PfEMP1 from rosetting *Plasmodium falciparum* parasites. Malaria Journal 2013 12:32.

IX: B. Small scale expression of soluble recombinant *Plasmodium vivax* proteins using the cell free wheat germ system and its use in Bioplex suspension array technologies

by ***Carmen Fernandez-Becerra[1], Edmilson Rui[1], Miriam Ramirez[1], Takafumi Tsuboi[2,3], and Hernando A. del Portillo[1,4]***

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This protocol provides a cost-effective 50 µl scale method for the expression of soluble *Plasmodium vivax* proteins using the cell free wheat germ system, for their purification and coupling to Bio-Plex magnetic beads and for their usage in multiplex immuno-epidemiological studies.

Equipment

EppThermomixer comfort (Eppendorf)
Centrifugue (4 °C refrigerated)
Sonicator
Vortex
Shaker
Vertical electrophoresis system (Bio-Rad)
Magnetic separator (DynaMag, Invitrogen)
Hand-held magnetic separation block(Millipore)
Bio- Plex 200 System (Bio-Rad)

Materials and Reagents

RTS 100 Wheat Germ CECF Kit (5 prime)
pIVEXGST1.4 vector [1]
GST SpinTrap(GE-HEALTHCARE)
Slide-A-Lyzer MINI Dialysis Unit (Piercenet)
PBS (10 mM Na_2HPO_4, 140 mM NaCl, 2.7 mM KCl, 1.8 KH_2PO_4, pH: 7.4)
Elution buffer (50 mM Tris-HCl, 10 mM reduced glutathione, pH 8.0)
Bio-Plex Amine Coupling Kit (Bio-Rad)
Bio -Plex COOH Bead(Bio-Rad)
Assay Buffer (PBS 1x, BSA 1%, Tween 20 0,02%, Sodium azide 0,02%)

Wash buffer [PBS 1x, Tween 20 0,05% (v/v)]
Bio-plex Calibration Kit (Bio-Rad)
Bioplex Validation Kit(Bio-Rad)
S-NHS(Sigma)
EDAC(Fisher)
Micro Assay plate 96 wells black (Greiner bio-one)

Procedure

Protein expression using Wheat Germ system, purification and dialysis (from 30 to 48 h)

- Clone the sequence of interest into the pIVEXGST1.4 vector in the correct reading frame. Use 2-4 µg of circular DNA in each expression reaction. This vector was obtained from the pIVEX1.4d vector (Roche) by inserting GST after the 6xHis tag sequence in the Ksp I site. Modified plasmid was termed pIVEXGST1.4 carrying the T7-DNA promoter element, the ampicillin selectable marker and His-GST tags [1].
- Reconstitute all the reaction components of the RTS 100 Wheat Germ CECF Kit and prepare feeding and reaction solutions following the manufacturers´ instructions.
- Place the microplate on the workbench and insert one or more CECF modules into it. Each CECF module contains two types of compartments (reaction compartments are labeled with red rings to distinguish them from feeding compartments, which are unlabeled).
- Pipette 1 ml feeding solution into each of the (unlabeled) feeding compartments, then pipette 50 µl reaction solution into each reaction compartment that is directly above a filled feeding compartment.
- Carefully close the modules of the microplate with the adhesive film (supplied in the kit), insert the microplate into the Eppendorf Thermo-mixer comfort and start the reaction following the next parameters (shaking speed: 900 rpm, temperature: 24°C and time: 24 h).
- After 24 h remove the solution (50 µl) from the reaction compartment, wash the reaction compartment with 50 µl of the feeding solution and mix, reaching a final volume of 100 µl. Store reaction solution frozen or at 4°C until purification or further processing.
- Purify the recombinant GST-tagged proteins using GST SpinTrap columns. Resuspend the resin in each GST SpinTrap column by inverting and shaking the column repeatedly. Loosen the top cap one-quarter of a turn and twist off the bottom closure. Place the column in a 2 ml microcentrifuge tube and centrifuge for 2 min at 100 × g to remove the storage liquid.
- Equilibrate the column by adding 600 µl of PBS. Centrifuge for 2 min at 100 × g and screw the bottom closure. Add to the sample, 100 µl of reaction solution plus 400 µl of PBS (maximum sample volume is 500 µl in one go) and incubate at 18 °C for 30 min with a rotation of 900 rpm (thermomixer) to ensure optimal binding of GST-tagged proteins to the Glutathione Sepharose 4B medium.
- Twist off the bottom closure and centrifuge for 2 min at 100 × g. Wash with 600 µl PBS and centrifuge for 2 min at 100 × g. Repeat the wash step once.
- Elute the target protein twice with 150 µl elution buffer (50 mM Tris-HCl, 10 mM reduced glutathione, pH 8.0). Add the elution buffer to the column and incubate at 18 °C for 15 min in the thermomixer at 900 rpm. Centrifuge for 2 min at 100 × g and collect the purified sample.
- The collected fraction containing the GST-tagged protein is dialyzed in 500 ml of PBS for 4 h in agitation at room temperature with a change of buffer every 2 h (or alternatively overnight at 4°C). Use the Slide-A-Lyzer MINI Dialysis Unit following the manufacture´s instructions.
- Determine the quality and concentration of the protein by SDS-PAGE and coomassie staining; use ImageJ software to quantify the amount of protein (http://rsb.info.nih.gov/ij/). Typically, 1-10 µg of soluble protein are obtained at 50 µl-scale.

Bio-Plex Protein Coupling and Validation (using Magnetic beads)

- For 1x scale coupling reaction activate 1.25 x 10^6 beads following the instructions provided with Bio-Plex Amine coupling kit.
- Add (2-4 µg) protein to the activated beads. Bring total volume to 500 µL with PBS, pH 7.4. Mix coupling reaction by vortex. Cover the coupling reaction tube with aluminium foil and incubate at 4ºC overnight with mixing on a shaker.
- Wash the beads with PBS following the instructions of the kit and place the tube into a magnetic separator and allow separation to occur for 30-60 sec. With the tube still positioned in the magnetic separator, remove the supernatant. Take care not disturb the beads.
- Resuspend the coupled and washed beads in 150 µL of storage buffer. Determine the bead concentration using a Coulter Z2 counter or haemocytometer. Store coupled beads refrigerated at 2–8°C in the dark.
- For protein coupling validation resuspend the coupled beads by vortexing at speed 7 for 30 sec and transfer desired volume of the stock beads (normally 2000 beads per well). Bring total volume to 50 µl/reaction with assay buffer. As all proteins expressed using this vector are GST-fusion proteins, we recommend to use GST as the “gold standard” for protein coupling validation [2].
- Dilute the antibodies with assay buffer and add 50 µl/well of the diluted primary antibody to the 50 µl of beads (final volume 100ul; final concentration 1:2000 to 1:4000).
- Cover the plate with adhesive film and incubate the reaction for 40-60 min at room temperature with mixing in a shaker at 800 rpm.
- Put assay plate containing magnetic beads on the hand-held magnetic separation block and secure the plate to the block using the block’s adjustable clip system. Allow the plate to sit on the block for 60-90 sec to allow settle by magnetic separator.
- Following the 60-90 sec bead settle, firmly grasp the base of the block in one of your hands over an appropriate waste receptacle. Next, in one fluid motion, turn the top of the plate so that it face the bottom of the waste receptacle, and move the block/plate in one swift, downward motion to discard the plate’s liquid contents into the waste receptacle.
- Gently tap the plate three to four times on absorbent pads to remove any residual liquid contents from the block. Using multi-channel pipette, load 100µL of wash buffer per well. Incubate the plate/block for 60 sec to allow bead settle by the magnetic separator and repeat the above steps, to discard the wash buffer. Repeat this step.
- Add 50µl/well of the diluted secondary biotinylated antibody (final concentration 1:4000 to 1:8000). Cover the plate with adhesive film and incubate the beads for 40-60 min as described previously.
- Repeat washed step as described previously and resuspend the beads in 50µl/well of Streptavidine-PE (1:1000) diluted in assay buffer. Incubate for 10 min at room temperature with mixing in a shaker at 800 rpm.
- Wash again and resuspend the beads in 125 µl of assay buffer for well. Shaker 1100 rpm at room temperature for 1 min.
- Proceed to read the coupled beads following the Bio-Plex protein array system operation.

References
Rui E, Fernandez-Becerra C, Takeo S, Sanz S, Lacerda MV, et al. (2011) *Plasmodium vivax*: comparison of immunogenicity among proteins expressed in the cell-free systems of Escherichia coli and wheat germ by suspension array assays. Malar J 10: 192.
Fernandez-Becerra C, Sanz S, Brucet M, Stanisic DI, Alves FP, et al. (2010) Naturally-acquired humoral immune responses against the N- and C-termini of the *Plasmodium vivax* MSP1 protein in endemic regions of Brazil and Papua New Guinea using a multiplex assay. Malar J 9: 29.

Figures

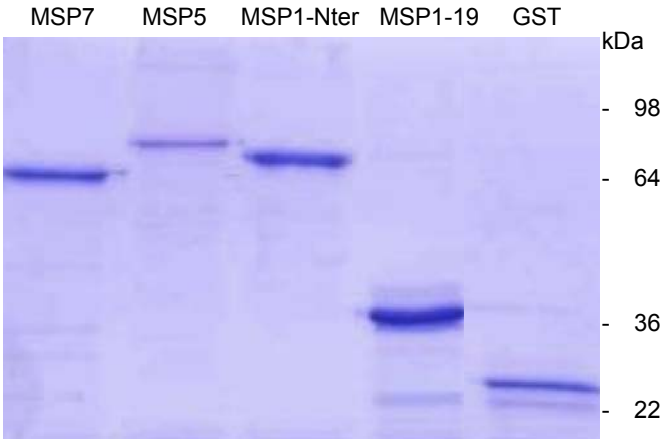

Figure 1. Analysis by SDS-PAGE and coomassie staining of *Plasmodium vivax* proteins produced at 50 µl-scale in the wheat germ cell-free system. Typically, between 1-10 µg of soluble protein are obtained at this scale. From 50 µl reaction were obtained between 1-10ug of protein. *Plasmodium vivax* proteins expressed: Merozoite surface protein 7 (MSP7), Merozoite surface protein 5 (MSP5), Merozoite surface protein 1 N-terminus (MSP1-Nter), Merozoite surface protein 1 C-terminus (MSP1-19), Gluthatione-S-transferase (GST). Molecular weights in kilo-Daltons are indicated to the right

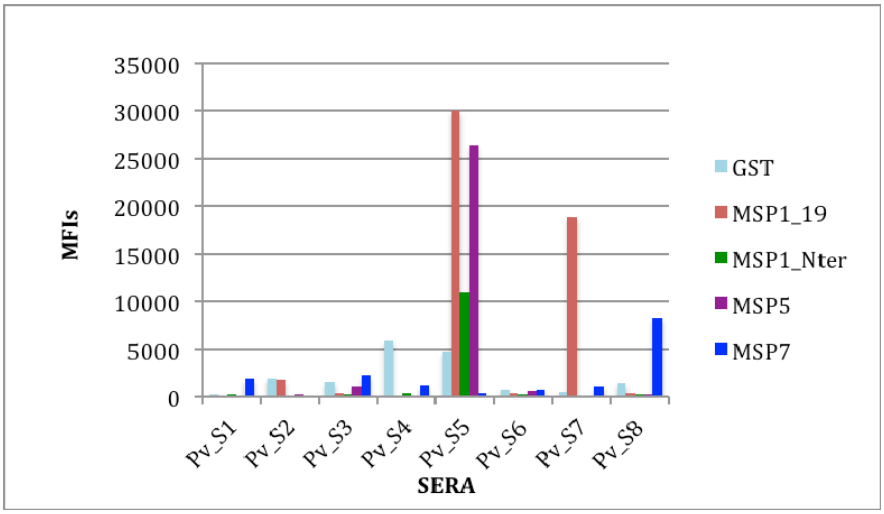

Figure 2. Human IgG antibodies against P. vivax recombinant proteins were detected by Bioplex. Pv_S1 – PvS8 refers to immune sera from individual P. vivax patients. Fluorescence was determined as the mean fluorescence intensity (MFI).

X. Telomere repeat amplification protocol in *Plasmodium falciparum* (PfTRAP)

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Equipment

homogenizer (Kontes; size 19)
slides and coverslips
optical microscope
centrifuges (15-mL tube and microfuge)
PCR machine
vertical electrophoresis system (10 × 8 cm)

Materials and reagents

parasite culture of 10% parasitemia containing ~10^9 parasites (mostly 16- to 30-h cultures)
Buffer B:
10 mM Tris–HCl (pH 7.5)
1 mM $MgCl_2$
1mM EGTA
5 mM 2-mercaptoethanol
10% glycerol
10 µg/mL leupeptin
10 µg/mL pepstatin
TRAP buffer:
200 mM Tris–HCl (pH 8.3)
15 mM $MgCl_2$
630 mM KCl
0.05% Tween 20
10 mM EGTA
500 µM dNTP
1 mg/mL BSA
Oligonucleotides:
PfTS (5′ AATCCGTCGAGCAGAGTTCA 3′)
PfCX (5′ GGCGCGT G/A AACCCT G/A AACCCT G/A AACCC 3′)
a GC-clamp followed by 3 telomere repeats)

Preparation

- Wash the homogenizer carefully and rinse it with 0.4 M NaOH, 0.25 M HCl, 0.4 M NaOH, and distilled water successively. Put it on ice before use.
- Use only RNase-free solutions and materials for the TRAP assay.
- Wear gloves all the time and keep parasite extracts on ice.

Preparation of a semipurified protein extract (~3 h)

- Do a saponin lysis of a freshly recovered culture (described elsewhere in this book) containing ~10^9 parasites.
- Wash the pellet in 5 volumes of PBS to eliminate the majority of erythrocyte contaminants.
- Wash the pellet in Buffer B.
- Resuspend the parasite pellet in 200 µL of Buffer B.
- Transfer parasites to the homogenizer and lyse the parasitophorous vacuole and cytoplasmic membrane by applying 80 strokes on ice.
- Carefully, take ~2 µL of the lysate and put it between the slide and a coverslip.
- Using a phase contrast optical microscope, check that the parasitophorous vacuole and cytoplasmic membrane have been disrupted (resulting in debris in the suspension). If not, apply 80 more strokes. Repeat this procedure until more than 90% of the parasites have been lysed.
- Transfer the lysate to a prechilled 1.5-mL tube.
- Centrifuge the lysate for 1 hr at 17,600 × *g* at 4 °C.
- The supernatant consists of a semipurified cytoplasmic fraction at a concentration of 5×10^6 parasite equivalents per microliter. Aliquot it in 2-, 4-, and 12-µL amounts and store it at −80 °C.
- The pellet containing the nuclei can be resupended in 200 µL of Buffer B, lysed by sonication, and centrifuged as described above.
- Both cytoplasmic and nuclear fractions contain telomerase activity. For practical reasons we usually use the cytoplasmic fraction.

TRAP assay (~6 h)

The TRAP assay consists of an elongation and an amplification step. In the elongation step, an oligonucleotide (PfTS) that mimics a chromosome extremity is used as a substrate for telomerase. The amplification step consists of a PCR in which the products from the elongation step are amplified and labelled with ^{32}P.

Elongation step:

- Use enough DNA in the reaction (equivalent to about 10^7 parasites) to get a positive reaction.
- In an RNase-free PCR tube, prepare the following mix:
  - 2 µL of protein extract
  - 5 µL of TRAP buffer
  - 100 ng of PfTS oligonucleotide
  - H_2O to 48.5 µL
- Incubate the mix at 37 °C for 1 h.

Amplification step:

- To each tube containing the elongation products add:
  - 100 ng of PfCX oligonucleotide
  - 2.5 µCi ^{32}P-dNTP
  - 2.5 U Ampli *Taq* Gold DNA polymerase (Applied Biosystems)
  - H_2O up to final volume of 50 µL
- Amplify as follows:
  - 1 cycle: 95 °C for 10 min
  - 35 cycles: 95 °C for 10 s, 55 °C for 30 s, 72 °C for 1 min

- Run 2 controls for nontelomerase-mediated incorporation. Before starting the elongation step, (i) pretreat semipurified protein extract for 30 min at 37 °C with 10 µg of RNase A and (ii) denature it at 95 °C for 10 min.
- Resolve PCR products on a 15% nondenaturing 8 × 10-cm polyacrylamide gel, at 150V (~2 h), in 1× TBE.
- At the end of electrophoresis, disassemble apparatus, leaving the gel stuck to one of the glass plates. Cover it with Saran wrap and expose it (without drying) to Kodak film for a few hours.

Reference

Bottius E, Bakhsis N, Scherf A. 1998. *Plasmodium falciparum* telomerase: de novo telomere addition to telomeric and nontelomeric sequences and role in chromosome healing. Mol Cell Biol 18(2):919-925.

XI. Genotyping of *Plasmodium falciparum* parasites

XI.A. Genotyping of *Plasmodium falciparum* parasites by PCR: *msp1*, *msp2*, and *glurp*

by ***Georges Snounou***[1] *and* ***Anna Färnert***[2]
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Equipment

micropipettes and tips
thermal cycler
apparatus for agarose gel electrophoresis
photographic equipment and UV transilluminator
refrigerator (4 °C) and freezer (−20 °C) for reagent storage
microcentrifuge

Materials and reagents

Taq polymerase (store at −20 °C) with appropriate buffer (store at 4 °C)
$MgCl_2$ stock solution (often provided by enzyme supplier)
dNTP: a working solution with a concentration of 5 mM for each of dATP, dCTP, dGTP and dTTP (store at −20 °C)
oligonucleotide primers (sequences are given below): a working solution with a concentration of 2.5 µM for each oligonucleotide primer (store at −20 °C)
mineral oil
loading buffer (5×):
50 mM Tris (pH 8.0)
75 mM EDTA (pH 8.0)
0.5% SDS
30% w/v sucrose
10% Ficoll (w/v, average molecular weight 400 000)
0.25% orange G dye (w/v approximately)
10× TBE buffer:
1 M Tris
1 M boric acid
50 mM EDTA
A pH of approximately 8.3 should be obtained without any adjustment.
Agaroses:
normal agarose
high resolution agaroses (such as NuSieve or MetaPhor, Cambrex)
ethidium bromide solution containing 10 mg per mL of water
CAUTION: This chemical is toxic and mutagenic. Wear protective clothing and use only in a chemical fume hood. Store in the dark at 4 °C.
pure water

Genotyping of *P. falciparum* infections permits characterization of distinct subpopulations present in an isolate. The genetic markers merozoite surface protein (*msp1*) (block2),

msp2, and glutamate rich protein (*glurp*) have been chosen due to their extensive polymorphism in size and sequences. The different allelic types, i.e., families, identified for *msp1* (K1, MAD 20, and RO33 types) and *msp2* (FC27 and IC/3D7) are detected with specific primers in a second nested PCR.

The amplification strategy used for genotyping *P. falciparum* parasites is the nested PCR. In the first amplification reaction oligonucleotide primer pairs, which will hybridize to conserved sequences flanking the repeat polymorphic regions of the genes, are used. The product of this first reaction is then used as a DNA template for separate second amplification reactions in which the oligonucleotide primers used recognise sequences contained within the DNA fragment amplified in the first reaction.

Setting up the first amplification reaction

- Calculate the total volume of reaction mixture required: 20 µL per reaction × (total number of reactions + 1).
- Calculate the amount of:
  buffer (final concentration 1×)
  $MgCl_2$ (final concentration 1.5 mM)
  dNTP (final concentration 125 µM)
  oligonucleotides (final concentration 250 nM)
  Taq polymerase (final concentration 2 units per 100 µL)
  and water to make up the total volume

NB: If all three genetic markers (*msp1*, *msp2,* and *glurp*) are to be analysed, then a mixture of the corresponding 3 oligonucleotide primer pairs can be added to the reaction. Thus one first amplification reaction only is required for each sample.

- Add, in order, the correct volumes of: water, buffer, $MgCl_2$, oligonucleotide primers, dNTP, and the *Taq* polymerase. Mix by a short vortex pulse.
- Aliquot 20 µL of the reaction mixture per labelled tube.
- Add 40 µL of mineral oil overlay to each tube.
- Add 1 µL of DNA template to each tube (water for the negative controls).
- Place in the cycler and run the PCR reaction.

Setting up the second amplification reactions

Steps 1 – 5 as above.

- Remove 1 µL from the first amplification reaction tube from under the oil overlay and add it to the oil overlay of the second amplification reaction tube.
- Place the tube in the cycler and run the PCR reaction.

NB: Each second amplification reaction must be performed with a single oligonucleotide pair. Thus, per sample, 3 separate reactions will be required for the analysis of *msp1,* two for *msp2,* and one for *glurp.*

Cycling parameters

Step 1	95 °C for 5 min	Initial denaturation
Step 2	**X** °C for 2 min	Annealing
Step 3	72 °C for 2 min	Extension
Step 4	94 °C for 1 min	Denaturation
Step 5	Repeat Steps 2–4 a total of 25 cycles (Nest 1) or 30 cycles (Nest 2)	
Step 6	**X** °C for 2 min	Final annealing
Step 7	72 °C for 5 min	Final extension
Step 8	The reaction is completed by reducing the temperature to 25 °C.	

X = 58 °C for Nest 1 (M1-OF/M1-OR; M2-OF/M2-OR; G-OF/G-OR) and the Nest 2 reactions of *glurp* (G-NF/G-OR).
X = 61 °C for Nest 2 reactions of *msp1* (M1-KF/M1-KR; M1-MF/M1-MR; M1-RF/M1-RR) and of *msp2* (M2-FCF/M2-FCR; M2-ICF/M2-ICR).

Sensitivity and specificity

- The parameters provided above (cycling parameters and final concentrations) may have to be altered in order to obtain optimal sensitivity and specificity. The use of different temperature cyclers and enzymes has an influence on the efficiency of the PCR reaction. The nested PCR for the 3 genetic markers should be capable of detecting about 10 parasite genomes per microliter of blood.
- Optimization of the PCR conditions requires the preparation of a standard set of genomic DNA templates. These are prepared from a defined quantity of in vitro cultured cloned parasite lines. Cloned lines must be used since the haploid genome contains a single copy of each of the 3 genes. If needed the conditions are varied in such a way that a single band is obtained for each of the specific second amplification reactions for a range of parasite genomic DNA concentrations.
- Sensitivity is mainly dependent on the number of cycles, thus these can be increased if needed.
- Specificity is most affected by the annealing temperature and eventually the $MgCl_2$ concentration. A common problem is the generation of two PCR products for the specific second amplification reactions. This is due to carryover of oligonucleotide primers and PCR product from the first reaction, which can be minimized by reducing the oligonucleotide concentrations and/or the number of cycles in the first amplification reaction.

Minimizing contamination

- The risks of contamination are enormously increased when nested PCR is performed. Thus the transfer of the product of the first amplification reaction to the second amplification reaction mixture should be performed with extreme care. In this context the oil that is used to overlay the reaction mixtures acts as a very efficient contamination barrier, and it is strongly suggested to retain the practice even when a heated lid is available.
- Ideally, setting up the first and second amplification reactions should be performed in a separate room from the one where the gels are migrated. Moreover, the transfer of the template from the first to the second amplification reactions must be performed with a dedicated pipette in yet another room, preferably with filter tips.

Analysis of the PCR product

- Add 5 µL of the loading buffer to the PCR product.
- Load 12 µL of sample on a suitable agarose gel and migrate (1× TBE buffer).
- Stain the gel in TBE buffer containing ethidium bromide (final concentration 1 µg per mL) for 30 min.
- Destain in TBE or water for 5 min.
- Visualise on a UV transilluminator.

Normal agarose is suitable for the analysis of all 3 genetic markers. However, given the small size of the bands which will result from the amplification of *msp1*, and the small variations in the sizes of the different allelic variants, the use of agarose type which give higher resolution is advised. The best results are obtained if the gel is kept cold before and during electrophoresis. The high cost of such agarose types (NuSieve or MetaPhor) can be compensated by the fact that the gels can be reused at least 10 times without significant loss of resolution. Used gels can be stored in TBE buffer, and when needed for further use, they are just reboiled. To compensate for condensation, add up water to the original gel volume when reboiling.

References

Arez AP, Lopes D, Pinto J, Franco AS, Snounou G, do Rosario VE. 2000. *Plasmodium* sp.: optimal protocols for PCR detection of low parasite numbers from mosquito (*Anopheles* sp.) samples. Exp Parasitol 94(4):269-272.

Färnert A, Arez AP, Babiker HA, Beck HP, Benito A, Björkman A, Bruce MC, Conway DJ, Day KP, Henning L, et al. 2001. Genotyping of *Plasmodium falciparum* infections by PCR: a comparative multicentre study. Trans R Soc Trop Med Hyg 95(2):225-232.

Färnert A, Arez AP, Correia AT, Björkman A, Snounou G, do Rosario V. 1999. Sampling and storage of blood and the detection of malaria parasites by polymerase chain reaction. Trans R Soc Trop Med Hyg 93(1):50-53.

Snounou G. 2002. Genotyping of *Plasmodium* spp. Nested PCR. Methods Mol Med 72:103-116. [This reference includes a detailed account of the protocol.]

Snounou G, Beck HP. 1998. The use of PCR-genotyping in the assessment of recrudescence or reinfection after antimalarial drug treatment. Parasitol Today 14(11):462-467.

Snounou G, Zhu X, Siripoon N, Jarra W, Thaitong S, Brown KN, Viriyakosol S. 1999. Biased distribution of *msp1* and *msp2* allelic variants *in Plasmodium falciparum* populations in Thailand. Trans R Soc Trop Med Hyg 93(4):369-374. (NB: The order of the msp1 K1 and MAD20 oligonucleotide was inverted in Table 1).

Oligonucleotide sequences

Merozoite Surface Protein 1: *msp1*

First reaction: M1-OF 5′- CTAGAAGCTTTAGAAGATGCAGTATTG -3′
M1-OR 5′- CTTAAATAGTATTCTAATTCAAGTGGATCA -3′

Second reaction:

K1-type M1-KF 5′- AAATGAAGAAGAAATTACTACAAAAGGTGC -3′
M1-KR 5′- GCTTGCATCAGCTGGAGGGCTTGCACCAGA -3′

MAD20-type M1-MF 5′- AAATGAAGGAACAAGTGGAACAGCTGTTAC -3′
M1-MR 5′- ATCTGAAGGATTTGTACGTCTTGAATTACC -3′

RO33-type M1-RF 5′- TAAAGGATGGAGCAAATACTCAAGTTGTTG -3′
M1-RR 5′- CATCTGAAGGATTTGCAGCACCTGGAGATC -3′

Merozoite Surface Protein 2: *msp2*

First reaction: M2-OF 5′- ATGAAGGTAATTAAAACATTGTCTATTATA -3′
M2-OR 5′- CTTTGTTACCATCGGTACATTCTT -3′

Second reaction:

FC27-type M2-FCF 5′- AATACTAAGAGTGTAGGTGCARATGCTCCA -3′
M2-FCR 5′- TTTTATTTGGTGCATTGCCAGAACTTGAAC -3′

IC/3D7-type M2-ICF 5′- AGAAGTATGGCAGAAAGTAAKCCTYCTACT -3′

M2-ICR 5′- GATTGTAATTCGGGGGATTCAGTTTGTTCG -3′

Glutamate Rich Protein: *glurp*
First reaction:G-OF 5′- TGAATTTGAAGATGTTCACACTGAAC -3′
G-OR 5′- GTGGAATTGCTTTTTCTTCAACACTAA -3′

Second reaction:
G-NF 5′- TGTTCACACTGAACAATTAGATTTAGATCA -3′
G-OR 5′- GTGGAATTGCTTTTTCTTCAACACTAA -3′

XI:B. *msp2* genotyping of *Plasmodium falciparum* by capillary electrophoresis and GeneMapper® Program

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Background

Genotyping by capillary electrophoresis and GeneMapper program.

The genotyping technique presented in the following characterises different *P. falciparum* clones by sizing the PCR product of the polymorphic marker gene merozoite surface protein 2 (msp2) using the GeneMapper® (formerly GeneScan®) program. This technique provides higher resolution of length polymorphic fragments and thus optimal discrimination of genetic diversity of the marker gene *msp2*.

The GeneMapper® program operates with data obtained with automated sequencer (3730xls DNA Analyser; Applied Biosystems Ltd). It measures the size of labelled DNA fragments by automated fluorescence detection after separating them by capillary electrophoresis technology. To overcome the problem of varying migration times between samples, an internal size standard is added, which is labelled with a different fluorescent dye than the sample PCR products. The size standard can be used to determine the length of the sample DNA fragments. It also serves as a positive control for the scanner analysis. The GeneMapper® system calculates the length of sample fragments based on the size standard length. Using the running times of fragments with known length, the program creates a calibration curve. For this the Local Southern Method is applied, where each point depends on the next two faster and the next two slower size standard fragments. The results of size calling can be displayed as electropherogrammes, as tabular data or as a combination of both. An alternative commercial program GeneMarker is available from http://www.gatc-biotech.com.

The latter can be used for analysing data from capillary electrophoresis or gels (e.g. PCR-RFLP.)

Previously reported applications of GeneScan®/GeneMapper® genotyping in *P. falciparum* includes microsatellite typing (Anderson et al. 2000) and *msp2* genotyping used to assess clonal population dynamics during malaria treatment (Jafari et al. 2004). Further discrimination of both allelic families of *msp2* has been described (Falk et al. 2006).

Msp2 genotyping.

Msp2 is the most size-polymorphic single copy locus of *P. falciparum*. In order to reach highest sensitivity of the assay, a primary PCR is followed by a nested PCR. For nPCR, fluorochrome-labelled reverse primers are used that are specific for either the 3D7 allelic family or the FC27 allelic family of *msp2*. Genotypes are distinguished by their fluorescent dye (indicating the allelic family) and by their size which is determined by an automated sequencer and GeneMapper® software.

Fluorescently labelled Primers.

As in most GeneMapper® applications, ROX-labelled size standards are used. The size standard used in this study consisted of 16 fragments ranging from 50 to 500 bp in length (Applied Biosystems). 6-FAM has been chosen for labelling

the FC27 family-specific primer M5. The 3D7 specific primer N5 was labelled with VIC. These two colours have been chosen because they have similar intensities and a big difference in emission wavelength.

Tailed Primers.

The tendency of the Taq polymerase to add a non-template nucleotide (usually an A) to the 3' end of the double-stranded DNA leads to PCR fragments which are 1 bp longer than expected. Because this happens only in a fraction of the reaction, a mixed population of fragments arises, and the precision of sizing is corrupted. A tailed forward primer (S_{Tail}) was used, which should promote the A-addition in nearly 100 percent, leading to a homogeneous population.

Equipment

thermocycler: any company such as MJ Research
(Optional: centrifuges for pre-PCR quick spin to collect mixture of master mix puls template at bottom of tube/plate: minifuge for tubes; if plates are used, a centrifuge with adaptor for plates is needed.)

Materials and reagents

PCR Reagents and materials

ddH_2O, home made, store at RT
10x Buffer B, Solis BioDyne, store at -20 ºC
dNTPs (Nucleotides) 2 mM, Qiagen, 201913, store at -20 ºC
$MgCl_2$ 25mM, Solis BioDyne, store at -20 ºC
FIRE Pol® DNA Polymerase I, Solis BioDyne, store at -20 ºC
Specific Oligos (Primer), ABI & Operon, store at -20 ºC
Tris EDTA concentrate (100x TE buffer), Fluka,86377, store at RT
mineral Oil[1], SIGMA, M5904, store at RT (not required if thermocycler with heated lid is used)
pipettes (1000 µL, 200 µL, 20 µL), Gilson, F267630, store at RT
500 µL reaction tubes, Sarstedt, 72.735.002, store at RT
filter tips 2-30 µL, F161933
filter tips 2-200 µL, F161934
filter tips 200-1000 µL, F161673, Gilson, store at RT
twin.tec PCR Plate 96,Eppendorf,0030 128.575 , store at RT
ROX-500 Size Standard,Applied Biosystems,401734, store at -20 ºC
deionised formamide (Hi-Di)
(Proposed supplier for reagents and consumables. Other products might be equally suitable.)

2. PCR primers

Primers used for primary PCR (according to Foley et al. 1992)

Primer	Sequence (5'→ 3')	Manufacturer
S2-fw	GAA GGT AAT TAA AAC ATT GTC	Operon
S3-rev	GAG GGA TGT TGC TGC TCC ACA G	Operon

PCR primers (Operon) are dissolved in 1x TE.
Prepare stock solution with a concentration of 100 µM.
For working solution, dilute stock solution to a concentration of 50 µM. Keep aliquots at -20 °C.
Primers used for nested PCR (labelled and unlabelled oligos according to Falk et al. 2006)

Primer	Sequence (5'→ 3')	Manufacturer
$S1_{Tail}$-fw	7bpTail - GCT TAT AAT ATG AGT ATA AGG AGA A	Applied Biosystems
M5-rev	6FAM - GCA TTG CCA GAA CTT GAA	Applied Biosystems
N5 -rev	VIC - CTG AAG AGG TAC TGG TAG A	Applied Biosystems

Primers used for nested PCR (labelled and unlabelled oligos according to Falk et al. 2006)

Primer	Sequence (5'→ 3')	Manufacturer
$S1_{Tail}$-fw	7bpTail - GCT TAT AAT ATG AGT ATA AGG AGA A	Applied Biosystems
M5-rev	6FAM - GCA TTG CCA GAA CTT GAA	Applied Biosystems
N5 -rev	VIC - CTG AAG AGG TAC TGG TAG A	Applied Biosystems

Post-PCR reagents and materials

ddH_2O, home made, store at RT
ROX-500 Size Standard, Applied Biosystems, 401734, store at -20ºC
pipettes (1000µL, 200 µL, 20µL), Gilson, F267630, store at RT
1.5 mL Safe-Lock tubes, Eppendorf, 0030 120.086, store at RT
filter tips 2-30 µL, F161933
filter tips 2-200 µL, F161934
filter tips 200-1000 µL, F161673, Gilson, store at RT
twin. tec PCR Plate 96, Eppendorf,0030 128.575 , store at RT.
PCR film, Eppendorf, 951023019, store at RT

Procedure

- Master mix primary PCR and primary PCR conditions
- Primary Mix:
  - ddH_2O 34.2 µL
  - 10x Buffer B (Solis) 5 µL
  - dNTPs (2 mM each) 5 µL
  - $MgCl_2$ (25mM) 3 µL
  - S2 Primer 50µM 0.25 µL
  - S3 Primer 50µM 0.25 µL
  - Taq polymerase (FirePol,Solis Biodyne, 5U/µl) 0.3 µL
  - **total volume 48 µL, add DNA 2 µL**

Thermoprofile primary PCR
94 ºC - 2 min

94 ºC - 30 sec
45 ºC - 45 sec 25 cycles*
70 ºC - 1 min 30sec

70 ºC - 10 min
* depending on efficacy of pPCR in a particular lab setting, number of cycles can be modified.

Master mix nested PCR and nested PCR conditions

- Nested Mix:
  ddH_2O 31.6 µL
  10x Buffer B (Solis) 5 µL
  dNTPs (2 mM each) 5 µL
  $MgCl_2$ (25mM) 3 µL
  Primer $S1_{Tail}$ (10 µM) 2 µL
  Primer N5 (10 µM) 1 µL
  Primer M5 (10 µM) 1 µL
  Taq polymerase (FirePol,Solis Biodyne, 5U/µl) 0.4 µL
  total volume 49 µL,
  add pPCR product 1 µL,

  Thermoprofile nested PCR
  94ºC - 2 min

  94 ºC - 30 sec
  50 ºC - 45 sec 25 cycles*
  70 ºC - 1 min 30 sec

  70 ºC - 10 min
  * depending on efficacy of nPCR in a particular lab setting, number of cycles can be modified.

- Prepare master mix for all samples to be amplified (plus positive and no template control) according to 3. or 4. in a template-free room dedicated to PCR with dedicated no template pipettes; use aerosol protected pipette tips. Aliquot master mix to reaction tubes or 96 well plate.
- Add DNA template or ddH_2O in case of no template control to master mix. Use a separate tip for each DNA sample. The template-adding step should be performed at a location different from that used for master mix preparation.
- If the thermo cycler used lacks a heated lid, overlay aliquoted master mix with 2 drops of mineral oil. In this case, the DNA template or pPCR product is added through the oil layer to the reaction mixture.
- Optional: To decrease risk of contamination, quick spin all tubes and plates containing extracted DNA or PCR product before opening tubes or removing caps or PCR film from plates.

- Post-PCR procedures
- Sample preparation for capillary electrophoresis:
- nPCR products (positivity checked on an agarose gel) are prepared for capillary electrophoresis as follows:
- 975 µl of ddH_2O are mixed with 25 µL of 8 nM size standard ROX-500 (1:40 dilution) and vortexed well. 10 µL of this solution are pipetted into each well of a 96 well plate, resulting in 0.25 µL of size standard per well.
- In a new 96 well plate 22.5 µL of water are pipetted in each well and 2.5 µL of nPCR product that have been found positive by gel electrophoresis are added in each well (1:10 dilution), the mixture is pipetted up and down several times to assure it is mixed well.
- 2.5 µL of the diluted PCR product are added to the size standard. The final amount of sample DNA corresponds to 0.25 µL PCR product per well and a size standard concentration of 0.16 nM. The solution is mixed by pipetting up and down several times.
- Prior to loading the automated sequencer 10 µL highly deionised formamide (Hi-Di) is added per well and carefully mixed. Then the plates are run on a 3730xls DNA Analyser (Applied Biosystems Ltd). The output files of the sequencer are in .fsa-format.

- Shipment of samples:
- If fragment sizing by capillary electrophoresis is not available, the plates are shipped to a provider of this service. The plate containing the correct dilutions of size standard and PCR product are put for air-drying in a dark box or drawer overnight. The next day, after all the liquid has evaporated, the plate is sealed with PCR film, labelled, covered with aluminium foil for protection against light and sent by mail.
- At the receiving end, the plates are spun down at 1650 g for a minute and stored at -20 °C until further processing. Prior to loading the automated sequencer, 10 µL highly deionised formamide (Hi-Di) is added per well and the samples are incubated at room temperature for 45 min.

- Analysis of samples with GeneMapper® Software:
- Electropherogrammes are analysed using the GeneMapper® program (Applied Biosystems Ltd.). Peaks are identified using the following parameters: Minimal peak width is set to 2 measurement points, the polynomial degree of the fitting curve is 3, and the peak window size is 15 measurement points. The sizes of the fragments are calculated by the local southern method, where each point is analysed with help of the next two points in either direction. The peak list generated by GeneMapper® is exported as tab delimited text for further analysis.

References

Anderson, T. J., B. Haubold, J. T. Williams, J. G. Estrada-Franco, L. Richardson, R. Mollinedo, M. Bockarie, J. Mokili, S. Mharakurwa, N. French, J. Whitworth, I. D. Velez, A.
H. Brockman, F. Nosten, M. U. Ferreira, and K. P. Day. 2000. Microsatellite markers reveal a spectrum of population structures in the malaria parasite Plasmodium falciparum. Mol. Biol. Evol. 17:1467-1482.

Falk N, Maire N, Sama W, Owusu-Agyei S, Smith T, Beck HP, Felger I. Comparison of PCR-RFLP and Genescan-based genotyping for analyzing infection dynamics of *Plasmodium falciparum*. Am J Trop Med Hyg. 2006 Jun;74(6):944-50.

Foley M, Ranford-Cartwright LC, Babiker HA.Rapid and simple method for isolating malaria DNA from fingerprick samples of blood. Mol Biochem Parasitol. 1992 Jul;53(1-2):241-4.
Jafari, S., J. Le Bras, O. Bouchaud, and R. Durand. 2004. *Plasmodium falciparum* clonal population dynamics during malaria treatment. J. Infect. Dis. 189:195-203.

XI:C. Genotyping of *Plasmodium falciparum msp1* and *msp2* using fluorescent PCR and capillary electrophoresis

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The protocol for genotyping of *P. falciparum msp1* or *msp2* by Snounou et al 1999 has been modified to fragment analysis by capillary electrophoresis (CE) (Liljander et al 2009). In the primary reaction, the primers used span the entire genetic segments i.e. block 2 for *msp1* and block 3 for *msp2* (Snounou et al). In the nested (second) reaction, the separate primer pairs targeting the respective allelic types of *msp1* (K1, MAD20, and RO33) and *msp2* (FC27 and IC) have been labeled with different fluorescent dyes and the reaction has been slightly modified. Fragment analysis is performed by capillary electrophoresis in a DNA sequencer which generates fragment lengths with base pair precision.

Reagents, concentrations and conditions presented here are recommendations however specific adjustments to different laboratory and equipment might be necessary.

Material and reagents

Equipment

Micropipettes: Different pipettes should be devoted to the different steps. Filter tips are not needed when preparing the PCR master. For the other steps it is advisable to use filter tips to avoid contamination of the pipettes.
Thermal cycler
DNA sequencer e.g. Applied Biosystems (ABI) 3730 or 3130xl sequencer or equivalent
Gene Mapper® software or equivalent

PCR and CE reagents and materials

Product	Company	Product no.
H_2O (PCR clean)		
AmpliTaq DNA Polymerase (5units/µl), 10x Buffer II and $MgCl_2$ 25mM	ABI	N8080153
dNTPs (nucleotides)	ABI	N808-0007
Hi-Di formamide	ABI	N4311320
GS LIZ 1200 (size standard)	ABI	4379950
96-well plate (compatible with the sequencer)		
POP-7 TM Performance Optimized Polymer	ABI	4335615 or 4363929
Genetic Analyzer 10X Running Buffer with EDTA	ABI	402824

Oligonucleotide primers for *msp1* and *msp2* genotyping and fluorescent modifications for the CE method

F= forward
R= reverse

In the primary reaction
msp1 F 5'-CTAGAAGCTTTAGAAGATGCAGTATTG-3'
msp1 R 5'-CTTAAATAGTATTCTAATTCAAGTGGATCA -3'
msp2 F 5'-ATGAAGGTAATTAAAACATT GTCTATTATA-3'
msp2 R 5'- CTTTGTTACCATCGGTACATTCTT-3'

Allelic type-specific primers for *msp1* in the nested reaction
K1 F 5'- AAATGAAGAAGAAATTACTACAAAAGGTGC-3' 7 bp-tail
K1 R 5'- GCTTGCATCAGCTGGAGGGCTTGCACCAGA-3' NED™ (yellow)
MAD 20 F 5'- AAATGAAGGAACAAGTGGAACAGCTGTTAC -3' 7 bp-tail
MAD 20 R 5'- ATCTGAAGGATTTGTACGTCTTGAATTACC'-3' PET® (red)
RO33 F 5'- TAAAGGATGGAGCAAATACTCAAGTTGTTG-3' 7 bp-tail
RO33 R 5'- CATCTGAAGGATTTGCAGCACCTGGAGATC-3' VIC® (green)

Allelic type-specific primers for *msp2* in the nested reaction
FC27 F 5'- AATACTAAGAGTGTAGGTGCARATGCTCCA-3' 7 bp-tail
FC27 R 5'- TTTTAT TTG GTGCAT TGCCAGAAC TTG AAC-3' 6-FAM™ (blue)
IC F 5'- AGAAGTATGGCAGAAAGTAAKCCTYCTACT-3' 7 bp-tail
IC R 5'- GATTGTAATTCGGGGGATTCAGTTTGTTCG-3' VIC® (green)

The primer pairs are ordered together; one labelled with fluorescent dye and one tailed (7 bp). It does not matter if the forward or reverse primer is labeled or tailed as long as the modification is put on the 5'-end of the primer. The 7 bp-tail is added by ABI at request.

Controls
Positive control: *Plasmodium falciparum* laboratory cultured lines that correspond to the three and two allelic types of *msp1* and *msp2* respectively e.g. K1 (*msp1* K1/*msp2* FC27), F32 (*msp1* MAD20/*msp2* IC) and 7G8 (*msp1* RO33/ *msp2* IC). When establishing and evaluating the CE method it is advisable to run DNA in a concentration gradient i.e. include a wide range of concentrations (1000, 100, 50, 10, 5, 1, 0.5 parasites/µl) to ascertain a reliable sensitivity. Moreover, mixing different lines in different ratios (e.g. 1000:10, 500:500, 10:1000) for PCR amplification is recommended to investigate the method sensitivity and specificity and for learning how to interpret the results. For routine amplifications it is advisable to use DNA in concentrations corresponding to 1000 parasites/µl and 50 or 10-25 parasites/µl as positive controls in each run.

Negative control: PCR master mix only or with added H_2O. Add 1 or more in every run. In a 96-well format, include at least 3 negative controls per plate, preferably randomly distributed over the plate.

PCR assay
Primary PCR reaction
Master mix final concentration per sample (20 µl);
1×PCR buffer
2 mM $MgCl_2$
125 µM dNTP (each)
0.02 units of AmpliTaq® DNA polymerase
250 nM each of the outer primer pairs; *msp1* forward (F)/reverse (R) and/or *msp2* F/R.

Add desired amount of DNA template (1-3 µl)
Nested PCR reaction (fluorescent)
Master mix final concentration per sample (20 µl);
1×PCR buffer
1 mM $MgCl_2$
125 µM dNTP (each)
and
125 nM each (F/R) primers of *msp1* (MAD20, K1 and RO33) and *msp2* FC27 in separate reactions
0.02 units of AmpliTaq® DNA polymerase in the *msp1* (MAD20, K1 and RO33) and *msp2* FC27 reactions
or
300 nM each (F/R) primers of the IC type
0.05 units AmpliTaq® DNA polymerase in the *msp2* IC type specific reaction

Add 1 µl primary PCR product as template in the respective nested reactions

NB: Each nested reaction has to be run separately, thus cannot be multiplexed.
Amplification conditions
The cycle conditions for the primary reaction:
- step 1- initial denaturation for 5 min at 95°C
- step 2- annealing for 2 min at 58°C
- step 3- extension for 2 min at 72°C
- step 4- denaturation for 1 min at 94°C, steps 2-4 were repeated 24 times
- step 5- final annealing for 2 min at 58°C
- step 6- final extension for 5 min at 72°C

Cycle conditions for the nested reactions:
- step 1- initial denaturation for 5 min at 95°C
- step 2- annealing for 2 min /1 min at 61° C /58°C for msp1 and msp2 respectively.
- step 3- extension for 2 min /1 min at 72°C
- step 4- denaturation for 1 min /30 sec at 94°C, steps 2-4 were repeated 22 times
- step 5- final annealing for 2 min/1 min at 61°C /58°C for msp1 and msp2 respectively
- step 6- final extension for 5 min at 72°C

Comments on the PCR assay
Keep the fluorescent primers in tubes wrapped with foil and avoid exposure to light. Once the primer pellets have been diluted the fluorescence will last for several months, perhaps longer. Fading of the color of the diluted primers is a sign of reduced fluorescence, which will be reflected by lower peak heights in the electropherogram.
The number of cycles in the nested reaction can be increased. In the present protocol, the number of cycles was reduced to reduce the appearance of stutters, but the cycle number can be optimized to the individual setting to increase the amplification of products and final peak height.

Fragment analysis by capillary electrophoresis
Capillary electrophoresis
Master mix final concentration per sample (9.5 µl);
HiDi formamide 9 µl
GS LIZ 1200 0.5 µl

Preparing the CE reaction
1. Dilute the nested PCR products e.g. 1:10 in H_2O, *see comment below*
2. Remove the HiDi from the freezer and let it thaw. Only thaw HiDi enough for the current run as HiDi is sensitive for freeze/thawing. When a new batch is bought, aliquot it into smaller volumes e.g. tubes with 900 µl that will be enough for one 96-well plate.
3. Add the calculated amount of GS LIZ 1200 and mix by careful vortexing.
4. Aliquot 9.5 µl CE master mix.
5. Add 1-2 µl diluted nested PCR product to each well, *see comment below*.
6. Check the plate for bubbles, *see comment below*.
7. Put on the septa and insert the plate in the cassette.
8. Put the plate into the sequencer and start the run.

Comments on fragment analysis by CE
In the CE step, the products can be "multiplexed" i.e. products from the separate nested reactions of for instance *msp1* i.e. K1, MAD20, RO33 from the same sample can be mixed. 1 µl of each product (in total 3 µl for *msp1*) will be mixed with 7 µl water. Then from this 1-2 µl diluted product will be added to the HiDi mix. Notice: Due to size competition between VIC-labeled products from *msp1* and *msp2* it is not recommended that *msp1* and *msp2* products are multiplexed.
If the nested PCR products are not diluted before CE the LIZ size standard will be affected and the sizes of those peaks will be substantially lower and thus affect the ability to use the standard. Increased amount of diluted PCR product added to the HiDi mix can increase the sample peak height. If you have any wells that do not contain sample in a row, these must be filled with 10 µl HiDi only. It is very important that the capillaries do not go dry and take up air. It is therefore also important to check that there are no bubbles in any of the wells. Keep the size standard refrigerated and dark at all time.

Analyzing the results using GeneMapper - how to interpret the results (from our experience)
To set up the analysis follow the instructions in the ABI manual (microsatellite analysis, getting started guide).

Cut off
To facilitate interpretation of the results a fluorescent cut off of 150 or 300 relative fluorescent units (rfu) is set. When a peak appears below cut off but looks like a true peak, the analysis should be rerun. The cut off can be set individually per fluorescent dye.

Stutters
Due to the repetitive structure of the *msp1* block 2 and *msp2* block 3 genes, stutter peaks (fragments lacking one or several repetitive units) can be detected along with the true fragment peak (Figure 1 and 2). Stutters have most commonly been seen for *msp2* FC27 allelic type; with stutter peaks situated 36-37 bp or 96-97 bp from the true allele peak (Figure 1) . The stutter peaks are often much lower than the true allele peak (about ~10% of the true allele peak height). Most often the stutter peaks are of similar height although with a slight decrease in height towards as they decrease in size. The fragments should not be considered as stutters if one peak in the "ladder" is missing and the visible peaks are of similar height as the true allele.

Stutters for the *msp2* IC type are less often detected, however when they appear the stutter-ladder most often look like the example below, with multiple low stutter peaks at the same intervals (12 bp distance in Figure 2) also substantially lower than the true allele peak.

Occasionally stutters at 27 bp and 9 bp intervals have been detected for the MAD20 type alleles. No stutters have been detected for the K1 and RO33 allelic types so far. However, one should always keep in mind that peaks that occur in a ladder at the same bp intervals might be stutters.

Figure 1

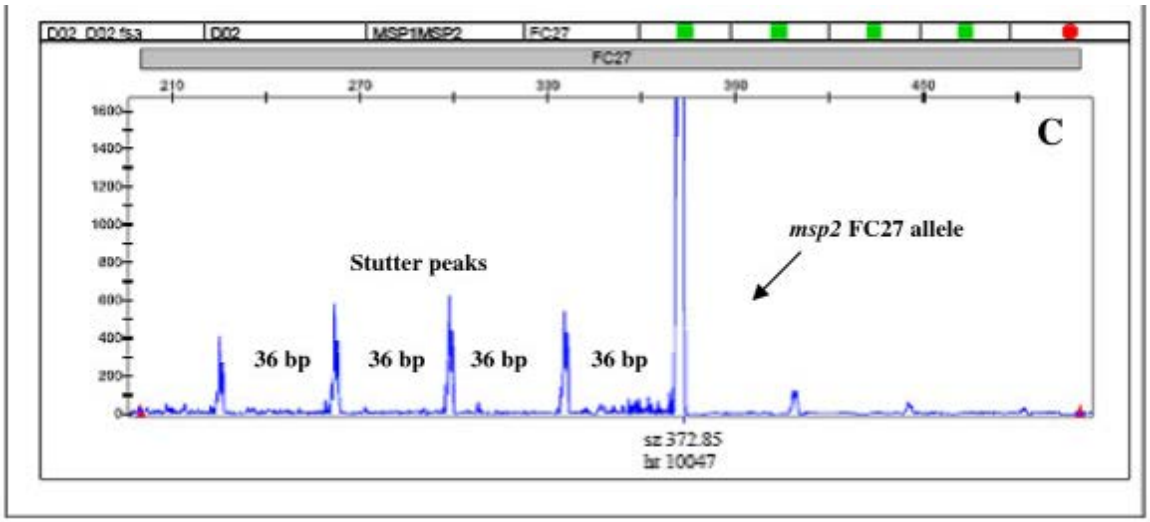
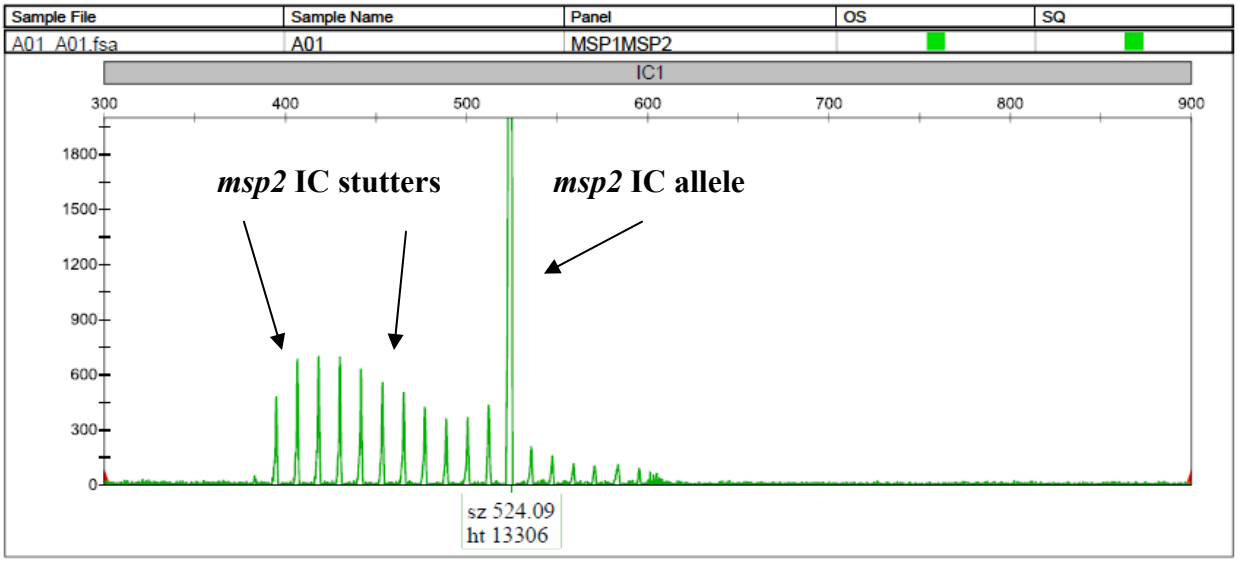
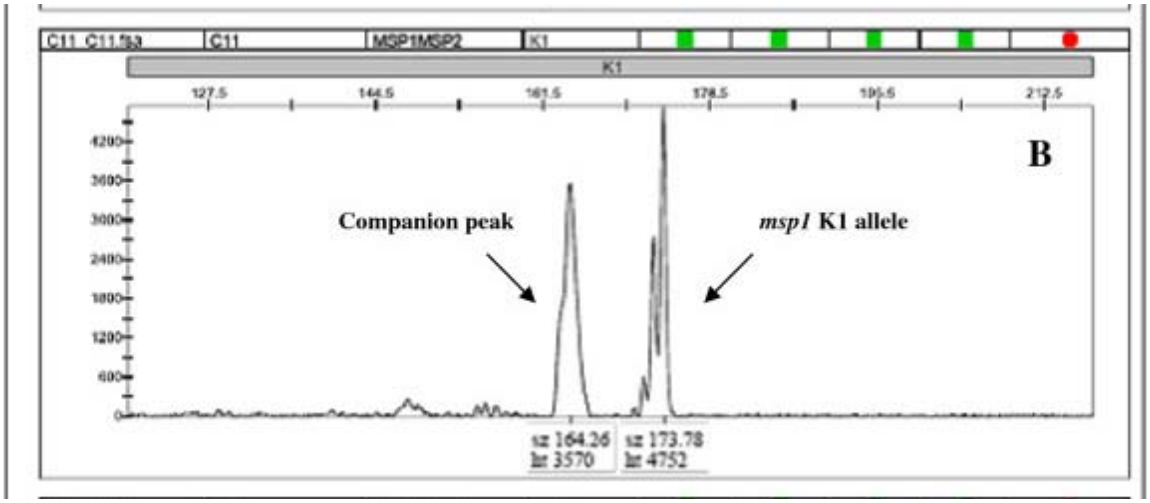


Figure 2



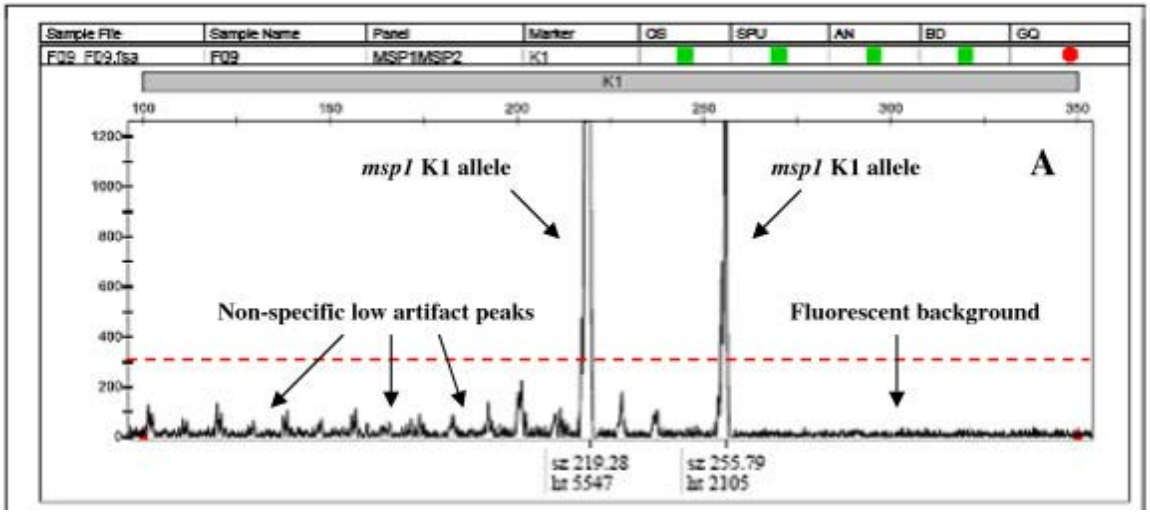
Companion peaks
Artifact peaks with a typical appearance occasionally occur at 5-20 bp to the left of the true peak (Figure 3). These peaks are wider and rounder and can often be of similar height or even higher than the true peak. These peaks are removed by diluting the PCR products in water before CE. The origin of these peaks is not known.

Figure 3



Non-specific low artifact peaks
Field samples sometimes give rise to non-specific low artifact peaks. These are however easy to disregard as they are below the cutoff.

Figure 4



References:

Snounou G, Zhu X, Siripoon N, Jarra W, Thaithong S, Brown KN, Viriyakosol S: Biased distribution of msp1 and msp2 allelic variants in *Plasmodium falciparum* populations in Thailand. *TransR Soc Trop Med Hyg* 1999, 93:369-374

Snounou G, Genotyping of *Plasmodium* spp.: Nested PCR. Methods in Molecular Medicine, Malaria Methods and Protocols: 2002, 72:103-116

Liljander A, Wiklund L, Falk N, Kweku M, Mårtensson A, Felger I, Färnert A. Multi-colored capillary electrophoresis for genotyping of *Plasmodium falciparum* merozoite surface proteins 1 and 2. *Malaria J 2009 8:78*

XII. Monitoring of malaria drug resistance associated SNPs in *P. falciparum* on microarray

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Equipment

PCR Thermocycler
incubator
centrifuge
microarray reader (microscopic slide format)
computer
12 or 8 channel pipette

Materials and reagents

QIAamp 96 DNA Blood Kit

96 well plates
10 x PCR Buffer
EDTA
dNTP mix containing all four nucleotides at 2mM conc.
25 mM $MgCl_2$
primer mixes for primary and nested PCR reactions
Taq Polymerase
thermosequenase
H_2O
ddNTP mixes (labelled with Cy3 and Cy5)
extension primer mix
Shrimp Alkaline Phosphatase
2x SSC + 0.1% SDS
2x SSC
2xSSC + 1% EtOH
10% SDS
costum made microarrays for drug resistance associated SNPs

Principle of procedure

In order to analyse all known single nucleotide polymorphisms associated with antimalarial drug resistance we have developed this microarray platform. The microarray is currently set up for the following SNPs:
Pfdhps 436, 437, 540, 581, 613, 640 645
Pfdhfr 16, 51, 59, 108, 164
Pfmdr1 86, 184, 1034, 1042, 1246
Pfcrt 72, 74,75, 76, 97,152, 163, 220, 271, 326, 356, 371
Pfatpase6 538, 574, 623, 683,769
This is essentially a nested PCR amplification of target sequences containing the SNPs in question, followed by shrimp alkaline phosphatase digestion to eliminate all dNTPs for the subsequent step of a one tube primer extension reaction with fluorochrome labelled ddNTPs over the SNP sites. Because most affordable microarray readers can only satisfactory discriminate two fluorochromes simultaneously, two assays with different dye combinations have to be set up. After the extension reaction, extended primers are hybridized to antisense primers deposited on glass microarrays. Slides are scanned with

a microarray reader and dedicated software which identifies wildtype, mutant or mixed infection for each of the SNP sites.
This technique allows the analysis of several hundred samples within a time period of few days at very affordable costs. This technique is not suited for individual sample analysis, and therefore this protocol is set up for at least 48 samples.

Procedure (Crameri et al. 2007)

- Prepare DNA from whole blood (anti-coagulated with EDTA) or red blood cell pellets using QIAamp® 96 DNA Blood Kit (Qiagen, Hombrechtikon, Switzerland) according to the manufacturer's instructions (http://www1.qiagen.com/literature/protocols/QIAamp96 DNABlood.aspx)

Amplification of target sequences

- Primary PCR: set up reactions in 96 well plates to 50 µL final volume per well
- prepare per well:
  30.0 µL H_2O
  5.0 µL 10 x PCR buffer (without $MgCl_2$)[1]
  5.0 µL dNTP mix (2mM each)
  6.0 µL 25mM $MgCl_2$
  1.0 µL Primary PCR primer mix (10 µM each)[2]
  0.5 µL Taq polymerase 5U/µl
- distribute 47.5 µl of the mix into each well
- add 2.5 µl DNA
- run thermocycler program
  96 °C180 sec

  96 °C 30 sec
  52 °C 90 sec
  72 °C 90 sec

  20 cycles for symptomatic samples
  25 cycles for asymptomatic samples

[1] see buffers and reagents
[2] Primary PCR primer mixes (sequence information: see primer table):
1.P 1-1(*pfmdr1* PCR I:P 1-1 for / P1-1 rev, 10 µM each in TE buffer)
2.P 3-1(*pfmdr1* PCR II:P 3-1 for / P3-1 rev, 10 µM each in TE buffer)
3.P 5-1(*pfdhfr* PCR:P 5-1 for / P5-1 rev, 10 µM each in TE buffer)
4.P 8-1(*pfdhps* PCR:P 8-1 for / P8-1 rev, 10 µM each in TE buffer)
5.P 10-1(*pfcrt* PCR I:P 10-1 for / P10-1 rev, 10 µM each in TE buffer)
6.P 11-1(*pfcrt* PCR II:P 11-1 for / P11-1 rev, 10 µM each in TE buffer)
7.P 12-1(*pfcrt* PCR III:P 12-1 for / P12-1 rev, 10 µM each in TE buffer)
8.P 16-1(*pfcrt* PCR IV:P 16-1 for / P16-1 rev, 10 µM each in TE buffer)
9.P 17-1(*pfATPase6* PCR:P 17-1 for / P17-1 rev, 10 µM each in TE buffer)
10.P 18-1(*pfcrt* PCR V:P 18-1 for / P18-1 rev, 10 µM each in TE buffer)

Nested PCR:

- transfer into new 96 well plate to 100 µl final volume
- prepare per well:
  60.0 µL H_2O
  10.0 µL 10 x PCR buffer (without $MgCl_2$)

10.0 µL dNTP mix (2mM)
12.0 µL $MgCl_2$ (25mM)
2.0 µL Nested primer mix (10µM each)[3]
1.0 µL Taq polymerase 5U/µl

- distribute 95 µL of the mix into each well
- add 5 µL DNA Primary PCR product
- run thermocycler program
  96 °C180 sec

  96 °C 30 sec
  52 °C 90 sec
  72 °C 90 sec

  20 cycles for symptomatic samples
  25 cycles for asymptomatic samples

[3] Nested PCR primer mixes (sequence information: see primer table):
11.P 1(*pfmdr1* PCR I:P 1 for / P1 rev, 10 µM each in TE buffer)
12.P 3(*pfmdr1* PCR II:P 3 for / P3 rev, 10 µM each in TE buffer)
13.P 5(*pfdhfr* PCR:P 5 for / P5 rev, 10 µM each in TE buffer)
14.P 8(*pfdhps* PCR:P 8 for / P8 rev, 10 µM each in TE buffer)
15.P 10(*pfcrt* PCR I:P 10 for / P10 rev, 10 µM each in TE buffer)
16.P 11(*pfcrt* PCR II:P 11 for / P11 rev, 10 µM each in TE buffer)
17.P 12(*pfcrt* PCR III:P 12 for / P12 rev, 10 µM each in TE buffer)
18.P 16(*pfcrt* PCR IV:P 16 for / P16 rev, 10 µM each in TE buffer)
19.P 17(*pfATPase6* PCR:P 17 for / P17 rev, 10 □M each in TE buffer)
20.P 18(*pfcrt* PCR V:P 18 for / P18 rev, 10 □M each in TE buffer)

SAP (Shrimp Alkaline Phosphatase) digest of PCR products

- pool 10 µL of each nPCR reaction into a single well of a new 96 well plate
- mix and centrifuge briefly (PCR pool plate).
- transfer 10 µL of each well of PCR pool plate into a new 96 well plate and add 90 µL of H_20 to each well.
- mix and centrifuge briefly. (PCR pool plate 1:10).
- add 7 µL SAP mix to each well of a new 96 well plate.
- add 5 µL DNA from PCR pool plate 1:10 in duplicate (thus each plate holds now DNA in duplicate from 48 samples).
  SAP mix:
  4.0 µl H_2O
  1.0 µl 10 x SAP buffer
  2.0 µl Shrimp Alkaline Phosphatase (SAP) 1U/µL
- run thermocycler program: SAP digest: 1 hour at 37 °C, inactivation of SAP digest: 15 min at 90 °C

Primer extension

- Preparation of ddNTP mix (2 combinations):
  Combination 1: ddATP Cy3, ddCTP Cy3, ddGTP Cy5, ddUTP Cy5

  Combination 2: ddUTP Cy3, ddCTP Cy3, ddATP Cy5, ddGTP Cy5

- Stock concentration of labelled ddNTPs is 100 µM (Perkin Elmer, Boston, USA)
- combine 25 µl 100 µM of each ddNTP stock (final conc of each ddNTP is 25 µM)
- add 900 µL TE buffer (final concentration of each ddNTP is 2.5 µM)
- make aliquots and store as 2.5 µM ddNTP mix at -20 °C

Preparation of extension primer mix (2 combinations)

- Combination 1: *Pfdhps* 437, 540, 581, 613, 640
  Pfdhfr 16, 51, 59, 108, 164
  Pfmdr1 86, 184, 1034, 1042
  Pfcrt 72, 75B1, 152, 271, 326, 326B, 356, 356B
  Pfatpase6 538, 769, 769B
- Combination 2: *Pfdhps* 436, 613B, 645
  Pfdhfr 108B, 164B
  Pfmdr1 1246
  Pfcrt 74, 76, 97, 163, 220, 371
  Pfatpase6 574, 623, 683
- Primer stock concentration is 10 µM in TE:
- add 2 µl of each primer stock to the respective combination
- add TE accordingly to obtain a 62.5 nM conc. of each primer
  for Combination 1 (25 x 2 µl = 50 µl): add 270 µl TE buffer
  for Combination 2 (15 x 2 µl = 30 µl): add 290 µl TE buffer
- store extension primer mixes at +4 °C

Extension reaction

- set up 2 primer extension mixes (final volume of 20 µL)
- prepare per well:
  1.6 µL H_2O
  2 µL Sequenase Buffer (Solis Biodyne Buffer + 2,5mM $MgCl_{2)}$
  2 µL Extension primer mix Combination 1 or Combination 2 (62.5 nM)
  2 µL ddNTP mix Combination 1 (2.5 µM)
  0.4 µL Thermo Sequenase (5U/µl)
- add 8 µl of extension mix (Combination 1 and Cobination 2) to the SAP digested PCR products in the SAP plate to a final volume of 20 µl (fill one row with Combination mix 1, and fill the next row with combination mix 2)
- run thermocycler program
  94 °C 60 sec

  94 °C 10 sec
  50 °C 40 sec
  35 cycles

Hybridization to microarray[4]

- pool extension reaction mixes Combination 1 and Combination 2 from each sample (final volume = 40 µL)
- add 6 µl of denaturing solution to a final volume of 46 µl:
  0.5 µL 0.5M EDTA pH 8.0
  2.0 µL 10% SDS

3.5 µL H_20

- incubate at 94 °C for 60 sec
- keep on ice for 2 min
- add 23 µL of combined extension reaction into 1 well of the microarray
- add 6 µL 20 x SSC to each well of the slide
- incubate microarray in a dark humid chamber at 50 °C for 60-90 min
- wash microarray with:
  2x SSC + 0.2% SDS: 10 min at room temperature (RT)
  2x SSC: 10 min at RT
  2x SSC + 2% EtOH: 2 min at RT
- dry microarray with compressed air
- store at RT in the dark until read
- [4] arrays are spotted on patterned microscopic slides (12 or 16 well slides) with aldehyde activated surfaces (Array-It, Telechem, Sunnyvale, USA). The spotting protocol is available on request from hans-peter.beck@unibas.ch

Data acquisition and analysis

- Microarrays can be scanned with any microarray scanner suitable for microscopic slides.
- aquire signals for Cy3 (wavelength: 532 nm)
- aquire signals for Cy5 (wavelength: 635 nm)
- store single signal or combined signal images as tif-files for further analysis
- name files with unique identification code for study, slide, experiment, and operator

tif-files can be analysed using commercially available software such as Axon GenePix Pro (www.axon.com).

Additional dedicated software to determine the appropriate genotypes for each SNP site including recognition of mixed infections can be obtained from hans-peter.beck@unibas.ch

Buffers, reagents, and oligonucleotides

Buffers

- 500 mM EDTA pH 8.0
- 180 mM phosphate buffer pH 8.0
- 20 x SSC pH 7.0
- 2 x SSC
- 2 x SSC + 0.2% SDS
- 2 x SSC + 2% EtOH
- 10% SDS
- 1 x TE buffer (= 10 mM Tris/HCl pH 8.0)
- 10 mM Tris/HCl pH 7.4
- Prepare all buffers/solutions according to the protocols in Sambrook et al. 1989
- Store all buffers at room temperature

Reagents

- 10 x PCR buffer (=buffer B) (Solis BioDyne, Tartu, Estonia)
- 25 mM $MgCl_2$ (Solis BioDyne, Tartu, Estonia)
- *Taq* polymerase (Firepol®; 5 U/µl) (Solis BioDyne, Tartu, Estonia)
- dNTP mix (2mM each) (Qiagen, Hilden, Germany): dilute 100 mM stock solutions 1:50 in 10 mM Tris/HCl pH 7.4
- 10 x SAP buffer (Amersham Biosciences)
- Shrimp Alkaline Phospothase (SAP; 1U/µl)(Amersham Biosciences)
- 10 x Sequenase buffer (Amersham Biosciences)
- Thermo Sequenase (Termipol®; 5 U/µl) (Solis BioDyne, Tartu, Estonia)
- Store all reagents at -20 °C

- Cy3 /Cy5 labelled ddNTP (Perkin Elmer)
- Store 100 µM ddNTP stock solutions at -80 °C
- Oligonucleotides for microarray spotting:

  500 µM stock solutions in 180 mM phosphate buffer pH 8.0 (aliquots at -20°C), all spotting oligos carry an amino C-7 linker at the 3' end (Operon, Cologne, Germany)

- Oligonucleotides for extension:

  100 µM stock solutions in TE buffer pH 8.0 aliquots at -20°C, HPLC purified (Operon, Cologne, Germany)

Oligonucleotide primer sequences (Operon Biotechnologies GmbH, Cologne, Germany)

Name	Locus / fragment	Sequence
	Primary PCR amplification	5' 3'
P5-for	*dhfr*	TTTATGATGGAACAAGTCTGC
P5-1 rev	*dhfr*	ATTCATATGTACTATTTATTCTAGT
P8-1 for	*dhps*	ATTTTTGTTGAACCTAAACGTGCTGTTCA
P8-1 rev	*dhps*	CTTGTCTTTCCTCATGTAATTCATCT
P1-1 for	*mdr1*, first fragment	TTAAATGTTTACCTGCACAACATAGAAAATT
P1-1 rev	*mdr1*, first fragment	CTCCACAATAACTTGCAACAGTTCTTA
P3-1 for	*mdr1*, second fragment	AATTTGATAGAAAAAGCTATTGATTATAA
P3-1 rev	*mdr1*, second fragment	TATTTGGTAATGATTCGATAAATTCATC
P10-1 for	*crt*, first fragment	TTGTCGACCTTAACAGATGGCTCAC
P10-1 rev	*crt*, first fragment	AATTTCCCTTTTTATTTCCAAATAAGGA
P18-1 for	*crt*, second fragment	ACTTTATTTGTATGATTATGTTC
P18-1 rev	*crt*, second fragment	TAACTGCTCCGAGATAATTGT
P11-1 for	*crt*, third fragment	ATTTACTCCTTTTTAGATATCACTTA
P11-1 rev	*crt*, third fragment	TTATATTTTTTAAAAACTATTTCCCTTG
P16-1 for	*crt*, fourth fragment	TCTGTTATTTTTATTTCTTATAGGCTAT

P16-1 rev	*crt*, fourth fragment	CTTGTATGTATCAACGTTTTTCATCC
P12-1 for	*crt*, fifth fragment	AGGAAATAAATATGGGAATGTTTAATTGA
P12-1 rev	*crt*, fifth fragment	TTCTAAGATAATATTTCCTACACGGT
P17-1 for	*ATPase6*	AATATTGTTATTCAGAATATGATTATAA
P17-1 rev	*ATPase6*	TGGATCAATAATACCTAATCCACCTA
	Nested PCR amplification	5’ 3’
P5 for	*dhfr*	ACAAGTCTGCGACGTTTTCGATATTTATG
P5 rev	*dhfr*	AGTATATACATCGCTAACAGA
P8 for	*dhps*	TTGAAATGATAAATGAAGGTGCTAGT
P8 rev	*dhps*	CCAATTGTGTGATTTGTCCA
P1 for	*mdr1*, first fragment	TGTATGTGCTGTATTATCAGGA
P1 rev	*mdr1*, first fragment	CTCTTCTATAATGGACATGGTA
P3 for	*mdr1*, second fragment	GAATTATTGTAAATGCAGCTTTA
P3 rev	*mdr1*, second fragment	GCAGCAAACTTACTAACACG
P10 for	*crt,* first fragment	CTTGTCTTGGTAAATGTGCTC
P10 rev	*crt* first fragment	GAACATAATCATACAAATAAAGT
P18 for	*crt*, second fragment	TCCTTATTTGGAAATAAAAAGGGAAATT
P18 rev	*crt*, second fragment	TAAGTGATATCTAAAAAGGAGTAAAT
P11 for	*crt,* third fragment	ACAATTATCTCGGAGCAGTTA
P11 rev	*crt*, third fragment	CATGTTTGAAAAGCATACAGGC
P16 for	*crt*, fourth fragment	CTTTTTCCAATTGTTCACTTCTTG
P16 rev	*crt*, fourth fragment	TCTTACATAGCTGGTTATTAAAT
P12 for	*crt*, fifth fragment	ACCATGACATATACTATTGTTAG
P12 rev	*crt*, fifth fragment	TTATAGAACCAAATAGGTAGCC
P17 for	*ATPase6*	AGCAAATATTTTCTGTAACGATAATA
P17 rev	*ATPase6*	TGTTCTAATTTATAATAATCATCTGT
	Extension primer at SNP site	5’ 3’
16	*dhfr* 16	GACGTTTTCGATATTTATGCCATATGTG
51	*dhfr* 51	GAAATAAAGGAGTATTACCATGGAAATGTA
59	*dhfr* 59	TTCACATATGTTGTAACTGCAC
108	*dhfr* 108 forward	CAAAATGTTGTAGTTATGGGAAGAACAA
108B	*dhfr* reverse	AAAGGTTTAAATTTTTTTGGAATGCTTTCCCAG
164	*dhfr* 164 forward	GGGAAATTAAATTACTATAAATGTTTTATT
164B	*dhfr* 164 reverse	TTCTTGATAAACAACGGAACCTCCTA
436	*dhps* 436	TTATAGATATAGGTGGAGAATCC

437	*dhps* 437 reverse	TTGGATTAGGTATAACAAAAGGA
540	*dhps* 540	AGGAAATCCACATACAATGGAT
581	*dhps* 581	GGATACTATTTGATATTGGATTAGGATTTG
613	*dhps* 613 forward	GGATATTCAAGAAAAAGATTTATT
613B	*dhps* 613 reverse	ATTTTGATCATTCATGCAATGGG
640	*dhps* 640 reverse	CAATTGTGTGATTTGTCCACAA
645	*dhps 645*	ATAAAAATATTGTGGACAAATCAC
86	*mdr1* 86	TTTGGTGTAATATTAAAGAACATG
184	*mdr1* 184	TGCCAGTTCCTTTTTAGGTTTAT
1034	*mdr1*1034	ATTGTAAATGCAGCTTTATGGGGATTC
1042	*mdr1* 1042 reverse	AGAAGGATCCAAACCAATAGGCAAAACTAT
1246	*mdr1* 1246	TAATATATGTGATTATAACTTAAGA
72	*crt* 72	TTTTAAGTATTATTTATTTAAGTGTA
76	*crt* 76 reverse	TTTGTTTAAAGTTCTTTTAGCAAAAATT
97	*crt* 97	GTTTTGTAACATCCGAAACTCA
152	*crt* 152	CCTTCATAGGTCTTACAAGAACT
163	*crt* 163	ATCCAATCATTTGTTCTTCAATTAAG
220	*crt* 220	TTCTATCATATTTAATCTTGTCTTAATTAGT
271	*crt* 271	TATACACCCTTCCATTTTTAAAA
326	*crt* 326	AAACCTTCGCATTGTTTTCCTTCTTT
326B	*crt* 326 reverse	ACATAGCTGGTTATTAAATTATCACAAATG
356	*crt* 356	TTGTTAGTTGTATACAAGGTCCAGCA
356B	*crt* 356 reverse	GGCTAAGAATTTAAAGTAATAAGCAATTGCT
371	*crt* 371	CTTTTTAATTTTATAGGGTGATGTTGTAA
538	*ATPase6* 538	AAATGTAATAAAGCTAATTCGGT
574	*ATPase6* 574	TGAAAAAAATACAACACCTGTAC
623	*ATPase6* 623	AACCATTCTAATTATACTACAGCTCAGG
683	*ATPase6* 683	TGAATGTATTTCTTCTTGGAGAAA
769	*ATPase6* 769	ACTTAGCTTTGCTTATAAAAAATTAA
	Arrayed as antisense oligonucleotides	5’ 3’ ---- C7
16 C-7	*dhfr* 16	CACATATGGCATAAATATCGAAAACGTC
51 C-7	*dhfr* 51	TACATTTCCATGGTAATACTCCTTTATTTC
59 C-7	*dhfr* 59	GTGCAGTTACAACATATGTGAA
108 C-7	*dhfr* 108 forward	TTGTTCTTCCCATAACTACAACATTTTG

108B C-7	*dhfr* reverse	CTGGGAAAGCATTCCAAAAAAATTTAAACCTTT
164 C-7	*dhfr* 164 forward	AATAAAACATTTATAGTAATTTAATTTCCC
164B C-7	*dhfr* 164 reverse	TAGGAGGTTCCGTTGTTTATCAAGAA
436 C-7	*dhps* 436	GGATTCTCCACCTATATCTATAA
437 C-7	*dhps* 437 reverse	TCCTTTTGTTATACCTAATCCAA
540 C-7	*dhps* 540	ATCCATTGTATGTGGATTTCCA
581 C-7	*dhps* 581	CAAATCCTAATCCAATATCAAATAGTATCC
613 C-7	*dhps* 613 forward	AATAAATCTTTTTCTTGAATATCC
613B C-7	*dhps* 613 reverse	CCCATTGCATGAATGATCAAAAT
640 C-7	*dhps* 640 reverse	TTGTGGACAAATCACACAATTG
645 C-7	*dhps 645*	GTGATTTGTCCACAATATTTTTAT
86 C-7	*mdr1* 86	CATGTTCTTTAATATTACACCAAA
184 C-7	*mdr1* 184	ATAAACCTAAAAAGGAACTGGCA
1034 C-7	*mdr1* 1034	GAATCCCCATAAAGCTGCATTTACAAT
1042 C-7	*mdr1* 1042 reverse	ATAGTTTTGCCTATTGGTTTGGATCCTTCT
1246 C-7	*mdr1* 1246	TCTTAAGTTATAATCACATATATTA
72 C-7	*crt* 72	TACACTTAAATAAATAATACTTAAAA
76 C-7	*crt* 76 reverse	AATTTTTGCTAAAAGAACTTTAAACAAA
97 C-7	*crt* 97	TGAGTTTCGGATGTTACAAAAC
152 C-7	*crt* 152	AGTTCTTGTAAGACCTATGAAGG
163 C-7	*crt* 163	CTTAATTGAAGAACAAATGATTGGAT
220 C-7	*crt* 220	ACTAATTAAGACAAGATTAAATATGATAGAA
271 C-7	*crt* 271	TTTTAAAAATGGAAGGGTGTATA
326 C-7	*crt* 326	AAAGAAGGAAAACAATGCGAAGGTTT
326B C-7	*crt* 326 reverse	CATTTGTGATAATTTAATAACCAGCTATGT
356 C-7	*crt* 356	TGCTGGACCTTGTATACAACTAACAA
356B C-7	*crt* 356 reverse	AGCAATTGCTTATTACTTTAAATTCTTAGCC
371 C-7	*crt* 371	TTACAACATCACCCTATAAAATTAAAAAG
538	*ATPase6* 538	ACCGAATTAGCTTTATTACATTT
574	*ATPase6* 574	GTACAGGTGTTGTATTTTTTTCA
623	*ATPase6* 623	CCTGAGCTGTAGTATAATTAGAATGGTT
683	*ATPase6* 683	TTTCTCCAAGAAGAAATACATTCA
769	*ATPase6* 769	TTAATTTTTTATAAGCAAAGCTAAGT

References

Crameri A, Marfurt J, Mugittu K, Maire N, Regös A, Coppee JY, Sismeiro O, Burki R, Huber E, Laubscher D, Puijalon O, Genton B, Felger I, Beck HP. (2007) A rapid microarray-based method for monitoring of all currently known single nucleotide polymorphisms associated with parasite resistance to antimalaria drugs. J. Clin. Microbiol. (*in press*).

Sambrook J., Fritsch E.F. and Maniatis T., *Molecular Cloning* (a laboratory manual; Sambrook J., Fritsch E.F. and Maniatis T. 2nd edition; Cold Spring Harbor Laboratory Press, 1989)

XIII. Nuclear run-on analysis

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Equipment

refrigerated centrifuge
dounce homogenizer (B-pestle)
hybridization oven
water baths/heating blocks (30ºC, 37ºC, 42ºC and 100ºC)
liquid scintillation counter
dot blot manifold (this protocol is for the 96-well manifold from Schleicher & Schuell)
pump to apply suction to the manifold

Materials and reagents

15 mL Falcon tubes
centrifugation tubes, resistant to organic solvents and 12 000g
Eppendorf tubes, RNase free
DEPC (diethyl pyrocarbonate)
0.1% Saponin in PBS (freshly made)
Triton X-100
100 µg yeast RNA
TRIzol (Gibco)
chloroform
isopropanol
75% Ethanol
Chromaspin columns (Chromaspin+TE-100; Clontech)
125 U/mL RnaseOut (Gibco)
100-150 µCi P^{32}-UTP 3000 Ci/mmol (Amersham Pharmacia)
DNase1 (RNase free!!) (Amersham Pharmacia)
DNA of interest, e.g. PCR products
Whatman papers, cut to fit the dot blot manifold
membrane, cut to fit the dot blot manifold (we recommend Hybond N^+ from Amersham)
1 M NaOH
200 mM EDTA pH8.2
ddH_2O
0.4 M NaOH

Buffer A:

10 mM Tris-HCl pH7.4
4 mM EDTA
15 mM NaCl
60 mM KCl

1 mM DTT
1 mM PMSF
0.15 mM Spermine
0.5 mM Spermidine

Buffer B:

50 mM Hepes pH7.9
50 mM NaCl
10 mM $MgCl_2$
1.2 mM DTT
10 mM Creatine phosphate
0.2 mg/mL creatine kinase
1 mM GTP and CTP
4 mM ATP
25% Glycerol

HSB (high salt buffer):
0.5 M NaCl
50 mM $MgCl_2$
2 mM$CaCl_2$
10 mM Tris-HCl pH7,4

Stop Buffer:
5% SDS
0.5 M Tris pH 7,4
0.125 M EDTA

Proteinase K
20 mg/mL in HSB buffer

Prehybridization solution:

50 mM Tris pH7,4
0.3 M NaCl
10 mM EDTA
0.2% SDS
1 mg/mL tRNA
0.5 mg/mL polyA
1% sodium pyrophosphate
5x Denhardts solution

Hybridization solution:

50 mM Tris pH7.4
0.3 M NaCl
10 mM EDTA
0.2% SDS
100 µg/mL tRNA

100 µg/mL polyA
0.1% sodium pyrophosphate
1x Denhardts solution

100x Denhardts solution:

20 mg/mL ficoll
20 mg/mL polyvinylpyrrolidone
20 mg/mL BSA

Wash solution 1:

2xSSC + 0.1% SDS

Wash solution 2:
0.1xSSC + 0.1% SDS

20x SSC :
3 M NaCl
0.3 M Na-Citrate

Procedure

- **Important: keep on ice at all times!!**

- Prepare all solutions with DEPC or DEPC-treated water. Use RNase-free plastic ware. Add CTP, GTP, ATP and Denhardts solution to their respective solutions before use. Prepare and add DTT, spermidine, spermine, PMSF, creatine kinase, creatine phosphate, Proteinase K, yeast RNA, tRNA, polyA and sodium pyrophosphate before use.

- **Nuclei isolation (all on ice!!!) and labeling**

  - Harvest cells from a *P. falciparum* culture. For the analysis of trophozoites and schizonts, 10 mL packed red blood cells with a parasitemia of > 5% are required and, for the analysis of rings, 20 mL of packed red cells with a parasitemia of > 10%.
  - Collect cells in 15 mL Falcon tubes on ice.
  - Discard supernatant.
  - Add 10 mL of 0.1% saponin (in PBS) to 1.5 mL of packed red blood cells. Erythrocytes are lysed by resuspension in saponin (0.1% w/v in PBS), permeabilizing both the host cell membrane and the parasitophorous vacuolar membrane (Benting et al. 1994; Ansorge et al. 1997; Allen and Kirk 2004).
  - Leave on ice until erythrocytes are completely lysed. It takes approximately 5 min.
  - Spin down at 2700 g for 8 min at 4 ºC.
  - Discard supernatant and wash pellet in buffer A (5-6 mL).
  - Spin down at 2700 g for 8 min at 4 ºC.

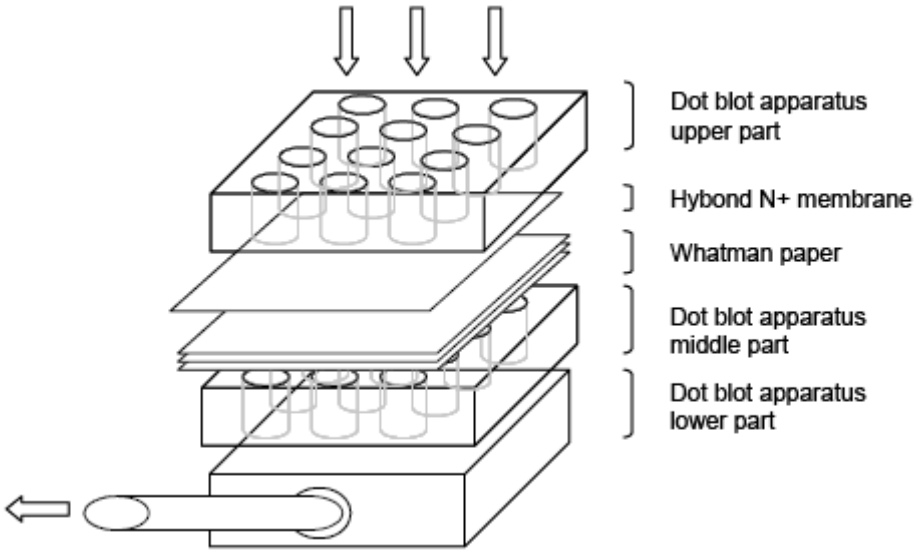
- Discard supernatant and resuspend liberated parasites in 2 mL of buffer A. Transfer the cell suspension to the Dounce homogenizer - on ice.
- Add 20 µL Triton X-100.
- To release nuclei, apply 10 strokes with the Dounce homogenizer.
- Spin at 12 000 g for 8 min at 4 ºC.
- Discard supernatant and wash pellet in 1 mL of buffer A.
- Spin at 12 000 g for 8 min at 4 ºC.
- Discard supernatant and resuspend pellet in 600 µL buffer B.
- Add 125 U/mL RNaseOut or other RNase inhibitor.
- Add 100 – 150 µCi of α-^{32}P UTP.
- Incubate for 30 min at 37ºC.
- Add 600 µL HSB and 10 U DNase.
- Incubate for 5 min at 30ºC.
- Add 10 µL proteinase K from a stock of 20 mg/mL.
- Incubate for 30 min at 42ºC.
- Add 200 µL Stop buffer

RNA isolation using TRIzol

- Add:
  100 µg yeast RNA
  4.5 mL TRIzol
  1.2 mL Chloroform
- Shake vigorously.
- Incubate for 5 min at room temperature.
- Spin down at 12000 g for 10 min at 4ºC.
- Transfer aqueous phase to a new tube.
- Add 3 mL isopropanol.
- Incubate for 10 min at room temperature.
- Spin at 12 000g for 10 min at 4ºC.
- Discard supernatant and wash pellet with 75% ethanol.
- Spin down at 7 000 g for 5 min at 4ºC.
- Discard supernatant carefully and air dry the RNA pellet for approximately 10 min.
- Resuspend RNA pellet in 70 µL H_2O (RNase free!).
- RNA is then size fractionated on a Chromaspin 100 column, which removes unincorporated label and RNA species smaller than 100 bp.
- To prepare the columns, spin for 5 minutes at 800g. Transfer isolated RNA carefully to columns, without letting the sample touch the sides of the column. Elute by centrifuging at 800g for 5 minutes.
- Measure the radioactivity of 1 µL in a liquid scintillation counter to ensure incorporation of α-^{32}P UTP. Usually 20.000 to 50.000 cpm are obtained per 1 µL.

Preparation of membrane

- Resuspend 1 pmol of double-stranded DNA in 100 μL of 0.4 M NaOH and 10 mM EDTA.
- Incubate at 100°C for 10 minutes and transfer to ice.
- Pre-wet the membranes in ddH_2O for 10 minutes.
- Pre-wet the Whatman papers in ddH_2O very shortly.
- Assemble the dot blot manifold, according to the manufacturer's recommendations (see figure)
- Add 400 µl ddH_2O to all the wells and apply vacuum pressure.
- Add the DNA to the wells and let it filter through (under suction). For orientation, add some colour, e.g. bromophenol blue, to some of the wells without DNA.
- Rinse the wells and membrane twice by adding 200 µl 0.4 M NaOH
- Let the membrane air dry. In a dry state, the membranes can be kept at room temperature until needed.
- Prehybridize membrane in prehybridization solution overnight at 65°C.



Hybridization and washing

- Add the labeled RNA to the hybridization solution (the smaller the volume the better), mix and add to the prehybridized membrane. Let hybridize over night at 42ºC.
- Wash membrane three times in wash solution 1 for 20 minutes at room temperature.
- Wash membrane twice for 20 minutes in wash solution 2 at 42°C.
- Analyse the results using a Phosphorimager or alternatively expose the membrane to a conventional X-ray film (at least over night exposure).

References

Allen, R. J. and K. Kirk (2004). "The membrane potential of the intraerythrocytic malaria parasite *Plasmodium falciparum*." J Biol Chem 279(12): 11264-11272.

Ansorge, I., K. Paprotka, S. Bhakdi and K. Lingelbach (1997). "Permeabilization of the erythrocyte membrane with streptolysin O allows access to the vacuolar membrane of *Plasmodium falciparum* and a molecular analysis of membrane topology." Mol Biochem Parasitol 84(2): 259-261.

Benting, J., D. Mattei and K. Lingelbach (1994). "Brefeldin A inhibits transport of the glycophorin-binding protein from *Plasmodium falciparum* into the host erythrocyte." Biochem J 300 (Pt 3): 821-826.

Lanzer, M., D. de Bruin and J. V. Ravetch (1992). "Transcription mapping of a 100 kb locus of *Plasmodium falciparum* identifies an intergenic region in which transcription terminates and reinitiates." Embo J 11(5): 1949-1955.

Schieck, E., Pfahler, JM., Sanchez, CP and M. Lanzer (2007). Nuclear run-on analysis of *var* gene expression in *Plasmodium falciparum*.
Mol Biochem Parasitol. 2007 Jun;153(2):207-12.

TRANSFECTION

I. Transfection of *Plasmodium falciparum* within human red blood cells

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There are 3 basic steps in asexual stage *P. falciparum* transfection:

A. Preparing the materials, parasite culture, and DNA constructs
B. Transfecting DNA into parasites by electroporation
C. Assaying the expression of transfected genes

This protocol includes the chloramphenicol acetyl-transferase (CAT) assay, luciferase assay, and the selection of stable transformants by the anti-folate pyrimethamine.

I:A. Materials

Parasite culture

Prepare 1×10^9 to 2×10^9 parasitized RBC (PRBC) for each transfection. A synchronized ring-stage parasite (<20 h) culture with high parasitemia (>15%) is essential for an improved transfection efficiency. For cultivation, synchronization, and purification see PARASITES, sections I through IV. Because washed human RBC deteriorate after 4 weeks at 4 °C (even sooner at 37 °C), the parasite culture should be replenished with fresh RBC before transfection, especially when a stable selection is desired.

DNA constructs

The design of transfection constructs is specific to the goal of each individual experiment. Plasmodial flanking regions are required for the expression in parasites; see also “References” at the end of this protocol. Use 50 to 100 μg of DNA for each transfection. The plasmid may be dissolved in TE buffer at 1 to 3 mg/mL.

Buffers

Incomplete Cytomix:
- 120 mM KCl
- 0.15 mM $CaCl_2$
- 2 mM EGTA
- 5 mM $MgCl_2$
- 10 mM K_2HPO_4/KH_2PO_4
- 25 mM HEPES

PBS:
- 1.7 mM NaH_2PO_4
- 5 mM Na_2HPO_4
- 150 mM NaCl

10% saponin/PBS stock solution

Equipment

GenePulser (Bio-Rad)
Capacitance Extender (Bio-Rad)
electroporation cuvettes (0.2-cm and 0.4-cm gap)
table-top centrifuge (Sorvall)

Reagents, buffers, and equipment for CAT assay

TEN:
1 mM EDTA
150 mM NaCl
40 mM Tris–HCl (pH 7.6)
250 mM Tris–HCl (pH 7.6)
[^{14}C]chloramphenicol (110 mCi/mmol, Moravek Biochemicals, diluted to 2.5 nCi/uL)
acetyl coenzyme A (20 mg, Calbiochem, dissolved in 250 mM Tris–HCl [pH 7.6] to 40 mM)
ethyl acetate
chloroform
methanol
TLC plate (aluminium-backed, thin-layer silica gel 60A plates, Whatman)
glass chromatography chamber
X-ray film and film cassettes, alternatively Phosphorimager cassettes and scanner (Molecular Dynamics)

Reagents, buffers, and equipment for luciferase assay

Luciferase Assay System (Promega):
cell lysis buffer
luciferase substrate
substrate solvent
purified luciferase as positive control
luminometer (Turner)

Reagents for stable selection

Pyrimethamine stock solution is made in dimethyl sulfoxide (DMSO, 50 mg/mL). Make the selection culture medium without gentamycin. The final content of DMSO in the culture medium should be less than 0.5%.

I:B. Transfection

Transfecting parasites, *Day 1*

- Change the parasite culture supernatant with fresh medium and make a smear to ensure the quality of the culture. Return the culture to the incubator for at least one h while preparing for transfection.
- Chill the electroporation cuvette and the Cytomix solution on ice. Set the GenePulser electroporator at 2.5 kV, 25 uF, 200 Ω.
- After everything is in place, spin down the cells at 1,500 rpm for 5 min in a table-top centrifuge. Resuspend the cells (about 100 to 200 μL) with 500 μL of Cytomix and not more than 100 μL of the DNA construct*. Mix well by pipetting up and down several times.

*Note: The DNA construct solution should be further concentrated if the volume to be added exceeds 100 μL in order to reach the desired final concentration in the transfection mix.

- Transfer the mixture into a 0.4-cm cuvette and execute the electroporation. Take a note of the set voltage, the actual voltage, capacitance, and time constant. A slight deviation of actual voltage is acceptable. The time constant should be 0.7 to 0.9 ms.
- Immediately return the cuvette to ice for 5 min, then transfer the cells from the cuvette to a culture flask together with 10 mL of culture medium (rinse the cuvette with culture medium to ensure a complete transfer). Return the flask to the incubator.
- An alternative electroporation setting is 0.31 kV/960 uF with the Bio-Rad GenePulser, using a 0.2-cm cuvette. The total volume allowed in the 0.2-cm cuvette is 400 μL. The time constant is about 12 to 15 ms.

Changing medium, *Day 2*

- Change the culture medium and add 50 μL of fresh blood. Check the parasite smear. Don't panic if the smear contains lots of extracellular, dying parasites or cell debris, as many parasites normally die during this procedure. Recovering parasites should have developed to trophozoites or schizonts by now.

Starting selection for stable transformants or changing medium for transient expression assay, *Day 3*

- **For stable transformation**, this is the day to start drug pressure. Feed the culture with medium containing pyrimethamine (100 ng/mL). On *Day 4*, continue to feed the culture with medium containing pyrimethamine (100 ng/mL). Most parasites die after 2 days of drug treatment, as can be seen in a Giemsa-stained thin smear. The pyrimethamine pressure may thus be dropped to 40 ng/mL. Continue this pressure until transformants are detected in Giemsa-stained thin smear (usually 3 to 5 weeks). Add 25 μL of fresh RBC into the culture each week during the selection procedure.
- **For transient CAT and luciferase expression assays,** continue to change the culture supernatant with 10 mL of fresh medium. Check the smear. New rings should emerge by *Day 3*.

I:C. Transfectant harvest and expression assays, *Day 4*

- **The luciferase assay** substrate is supplied as a powder and accompanied by a vial of solvent. Once the powder is dissolved, aliquot the substrate solution and store it at −20 °C. Before harvesting the cells, thaw and equilibrate the substrate to room temperature. Transfer 100 μL of the substrate into an assay tube and cover it with a piece of aluminium foil to avoid light.
- Spin down the parasite culture, resuspend the pellet in 1 mL of PBS and transfer it into a microfuge tube. Add 10 μL of 10% saponin/PBS stock to bring the final concentration of saponin to 0.1%; incubate the culture at room temperature for 5 min. Spin it at top speed in a microfuge for 3 min and aspirate the supernatant and RBC ghosts.
- Wash the pellet 2 or 3 times with PBS to remove hemoglobin which interferes with the luciferase assay. Resuspend the pellet completely in 30 μL of 1× lysis buffer by pipetting up and down. Freeze/thaw the sample for one cycle, then vigorously

pipette up and down again. The lysis of parasites is hardly discernible by eye because a large portion of the pellet is hemozoin. Centrifuge the lysate in a microfuge for 3 min at top speed. Take 20 μL of the supernatant for the luciferase assay.

- Because there is a slight delay of the photon peak after luciferase is added to the substrate, the timing of sample measurement is crucial if a quantitative comparison is desired. A practice optimized for Turner Luminometer measurements is to wait until the Luminometer is available (you are the only user at the time) and ready (the baseline has returned to zero). Set the timer for 10 s upon mixing the substrate and the lysate. Insert the tube into the chamber and start the measurement when the timer goes off. The set-up of the Turner Luminometer is usually done by a specialist from the vender company. This measuring protocol should be revised if a different luminometer is used.
- An alternative positive control for the luciferase assay is *Escherichia coli* cells carrying the tester plasmid.
- For the **CAT assay**, spin down the parasite culture, resuspend the pellet in 1 mL of PBS and transfer it to a microfuge tube. Add 10 μL of 10% saponin/PBS stock to make the final concentration to 0.1% and incubate the pellet at room temperature for 5 min. Spin it at top speed in a microfuge for 3 min, then aspirate the supernatant and RBC ghosts. Wash the pellet once with 1 mL of PBS and once with 1 mL of TEN. Resuspend the cell pellet in 120 μL of 250 mM Tris–HCl (pH 7.6) with PMSF (1 mM); pipette up and down vigorously. Freeze/thaw the sample for 3 cycles. The lysis of parasites is hardly discernible by eye because a large portion of the pellet is hemozoin. Centrifuge the lysate in a microfuge for 3 min at top speed. Incubate the lysate for 10 min at 60 °C to inactivate cellular acetylases. Clear the lysate by centrifugation in a microfuge at 15,000 × *g* for 2 min.
- In a microfuge tube, mix 10 μL of lysate, 20 μL of acetyl-CoA (40 mM), and 1 μL of [^{14}C]chloramphenicol (2.5 nCi). Incubate the mixture at 37 °C for 3 h, then add a second 20-μL aliquot of acetyl-CoA. Continue the incubation overnight.
- Handle all the samples, reagents, and waste according to radiation safety regulations from this point onward.

Continue the CAT assay, *Day 5*

- Momentarily spin down condensations of the reaction mix, then add 350 μL of ethyl acetate. Vortex to thoroughly mix and spin briefly to separate the organic and aqueous phases. Transfer 300 μL of the upper organic layer to a clean microfuge tube and dry the extract in a speed-vac centrifuge (30 to 60 min at 45 to 60 °C).
- Redissolve the sample with 25 μL of ethyl acetate. Mark the origin of a thin-layer chromatography (TLC) plate (Whatman) with a soft lead pencil 1.5 to 2 cm from the edge. Apply 10 μL of sample to the origin. Prepare a TLC chamber containing 200 mL of chloroform:methanol (20:1). Place the TLC plate in the chamber and allow the solvent front to move ~15 cm. Remove the plate from the chamber and air-dry the plate.
- Place the TLC plate in a film cassette and align the plate and the X-ray film with radioactive ink. A 3- to 5-day exposure is required due to the low level expression (or low transfection efficiency). Develop the film and align it with the plate. Alternatively, expose the TLC plate to a Phosphorimager screen for overnight and

scan it with the Phosphorimager. Three radioactive spots should be observed. The two faster migrating spots represent the 1- and 3-acetylated chloramphenicol. The spot that has migrated the least distance from the origin represents the nonacetylated form.

- Liquid scintillation may be used to quantify the CAT activity. This involves cutting the radioactive spots from the plate for measurement of the amount of radioactivity they contain.

References

Crabb BS, Triglia T, Waterkeyn JG, Cowman AF. 1997. Stable transgene expression in *Plasmodium falciparum*. Mol Biochem Parasitol 90(1):131-144.

Goonewardene R, Daily J, Kaslow D, Sullivan TJ, Duffy P, Carter R, Mendis K, Wirth D. 1993. Transfection of the malaria parasite and expression of firefly luciferase. Proc Natl Acad Sci USA 90(11):5234-5236.

Mamoun CB, Gluzman IY, Goyard S, Beverley SM, Goldberg DE. 1999. A set of independent selectable markers for transfection of the human malaria parasite *Plasmodium falciparum.* Proc Natl Acad Sci USA 96(15):8716-8720.

van der Wel AM, Tomas AM, Kocken CH, Malhotra P, Janse CJ, Waters AP, Thomas AW. 1997. Transfection of the primate malaria parasite *Plasmodium knowlesi* using entirely heterologous constructs. J Exp Med 185(8):1499-1503.

van Dijk MR, Janse CJ, Waters AP. 1996. Expression of a *Plasmodium* gene introduced into subtelomeric regions of *Plasmodium berghei* chromosomes. Science 271(5249): 662-665.

van Dijk MR, Vinkenoog R, Ramesar J, Vervenne RA, Waters AP, Janse C. 1997. Replication, expression and segregation of plasmid-borne DNA in genetically transformed malaria parasites. Mol Biochem Parasitol 86(2):155-162.

van Dijk MR, Waters AP, Janse CJ. 1995. Stable transfection of malaria parasite blood stages. Science 268(5215):1358-1362.

VanWye JD, Haldar K. 1997. Expression of green fluorescent protein in *Plasmodium falciparum*. Mol Biochem Parasitol 87(2):225-229.

Wu, Y, Kirkman LA, Wellems TE. 1996. Transformation of *Plasmodium falciparum* malaria parasites by homologous integration of plasmids that confer resistance to pyrimethamine. Proc Natl Acad Sci USA 93(3):1130-1134.

Wu Y, Sifri CD, Lei HH, Su XZ, Wellems TE. 1995. Transfection of *Plasmodium falciparum* within human red blood cells. Proc Natl Acad Sci USA 92(4):973-977.

II. Protocol using DNA-loaded red blood cells for transfection

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Equipment

Electroporator (Bio-Rad)

Materials and reagents

Incomplete Cytomix (see Wu et al. 1995):

8.95 g KCl
0.017 g $CaCl_2$
0.76 g EGTA
1.02 g MgC_2
0.871 g K_2HPO_4
0.68 g KH_2PO_4
7.08 g HEPES
Dissolve ingredients in water, adjust pH to 7.6 by adding NaOH, and adjust final volume to 1 liter. Filter-sterilize and use.

Preparation

The procedure outlined below is a modification of the above protocol and takes advantage of the observation that malaria parasites actively take up DNA from the cytoplasm of DNA-loaded red blood cells.

- Using a stock of red blood cells stored at 50% hematocrit, transfer 350 μL of cells to a 10- or 15-mL centrifuge tube and spin them down. Remove the supernatant and wash the pellet with 5 mL of Incomplete Cytomix. Spin down the cells again and remove the supernatant.
- Resuspend the cells in Cytomix containing 50 to 100 μg of plasmid DNA. The final volume should be 400 μL. Transfer the cells to a 0.2-cm cuvette and place it on ice.
- Electroporate the cells using the conditions outlined above. We are using the following conditions: voltage: 0.31 kV; capacitance: 960 μFD; time constants should be in the 10 to 14 ms range.
- Rinse the cells from the cuvette with 5 mL of culture medium. Two consecutive washes of 2.5 mL each works well. Transfer the cells to a centrifuge tube.
- Spin down DNA-loaded red blood cells and remove the supernatant. The supernatant should be quite red from cell lysis, however if nearly all of the red cells have lysed, check the pH of the Cytomix. It should be ~7.6.
- Transfer the cells to a culture flask and inoculate with parasites. Typically the red cells from two electroporations are used for a 5-mL culture. Parasites will take up and express DNA as they invade the DNA-loaded red blood cells.

Advantages of using DNA-loaded red blood cells

- Flasks containing DNA-loaded red blood cells can be inoculated with 100 to 200 μL of an established culture. Such parasites tend to be healthier because they have not been electroporated and give higher levels of transient expression compared to the same starting numbers of parasites transfected directly by electroporation.

- High levels of expression can be obtained by first loading red cells as described above, then inoculating the cells with mature stage parasites purified by Percoll/sorbitol gradients as outlined in PARASITES, section IV:D. As the cells infected by the mature stages rupture, the resulting merozoites invade the DNA-loaded red blood cells resulting in very high numbers of synchronous, transfected parasites.
- If extremely high levels of transient expression are desired, a culture of parasites can be continuously maintained with DNA-loaded red blood cells. After four generations (8 days) of parasite multiplication in DNA-loaded cells, maximal levels of expression are observed. These levels can be up to 20-fold higher than that observed after direct electroporation of parasite-infected cells.

References

Deitsch K, Driskill C, Wellems T. 2001. Transformation of malaria parasites by the spontaneous uptake and expression of DNA from human erythrocytes. Nucleic Acids Res 29(3):850-853.
Deitsch KW, Calderwood MS, Wellems TE. 2001 Cooperative silencing elements in var genes. Nature 412(6850):875-876.
Wu Y, Sifri CD, Lei HH, Su XZ, Wellems TE. 1995. Transfection of *Plasmodium falciparum* within human red blood cells. Proc Natl Acad Sci USA 92(4):973-977.

III. Selectable episomal transfection of asexual stage *Plasmodium falciparum* parasites

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Equipment
Gene Pulser electroporator with Capacitance Extender (Bio-Rad)
electroporation cuvettes (0.2-cm gap, Bio-Rad)
centrifuge
incubator (37 ^{o}C)
microscope with 100X lens
modular incubator chambers (Billups–Rothenberg)

Materials and reagents for plasmid preparation
electrocompetent or chemically competent *Escherichia coli* (We prefer XL10-Gold or DH5α for propagation of plasmids containing *P. falciparum* DNA)
incomplete Cytomix buffer:
120 mM KCl
0.15 mM $CaCl_2$
2 mM EGTA
5 mM $MgCl_2$
10 mM K_2HPO_4/KH_2PO_4
25 mM HEPES
Plasmid Maxiprep columns (Qiagen)

Materials and reagents for *P. falciparum* propagation
P. falciparum strains
gas mixture of 5% O_2, 5% CO_2, and 90% N_2
human red blood cells (RBC) washed free of leukocytes
RPMI 1640 with L-glutamine (Invitrogen, Catalog No. 31800)
hypoxanthine (Sigma)
HEPES (Sigma)
sodium bicarbonate (Invitrogen)
gentamycin (Invitrogen)
Albumax (I or II, Invitrogen)
complete medium:
RPMI 1640 with L-glutamine
50 mg/liter hypoxanthine
10 mg/liter gentamycin
25 mM HEPES
0.225% $NaHCO_3$
0.5% Albumax
Parafilm
sterile 6-well tissue culture plates
sterile glass pipettes and other tissue culture plastic ware
WR99210 (available upon request from David Jacobus, Jacobus Pharmaceuticals, Princeton, NJ, USA, e-mail: dpjacobus@aol.com)
blasticidin S HCl (Sigma)

atovaquone (Sigma)
G418 (Cellgro, Manassas, CA)

This protocol describes fundamental steps for selecting episomally transfected asexual-stage *P. falciparum* parasite lines:

A. Preparation of parasite cultures and DNA constructs
B. Electroporation of circular plasmid DNA into parasites
C. Maintenance of transfected parasite lines

A. Preparation of parasite cultures and DNA constructs

Parasite cultures

For each transfection, it is best to use 2.5 – 3 ml of a healthy culture at 4-8% parasitemia (predominantly ring stage parasites) and 3-5% hematocrit. On the day of transfection, replace the media with fresh media and determine parasitemia and predominant stage by microscopy (smear and giemsa stain). At the time of transfection, it is recommended that parasites are growing in fresh blood (i.e. RBCs that are within 1 week old). We have observed variation in the competence of different strains of *P. falciparum*. In general, Dd2 parasites effectively take up plasmid while 3D7 parasites are more difficult to transfect.

DNA constructs

Prepare pure supercoiled plasmid DNA using Maxiprep columns. Dissolve the DNA in Cytomix at a concentration of 0.5 to 2 mg/mL. Take care not to dry the DNA for an excessive amount of time (i.e. more than 10 minutes) prior to resuspending it in cytomix since this can reduce the solubility of the DNA in cytomix. If DNA does not dissolve easily in cytomix, this can be resolved by adding more cytomix (take care not to make the plasmid DNA too dilute) or gently heating the DNA at 40 °C followed by pipetting to resuspend the DNA. Fifty to 100 μg of plasmid DNA will be used per transfection.

The design of the construct and the choice of selectable marker can be altered depending on the desired final outcome. Plasmids are most often designed to achieve gene disruption (“knockout”) or allelic replacement (e.g., to exchange polymorphic codons). Selectable markers established for stable episomal transfection of *P. falciparum* include, human dihydrofolate reductase (*dhfr*, with or without the gene encoding green fluorescent protein as a fusion product), blasticidin S deaminase (from *Aspergillus terrus*), neomycin phosphotransferase II (transposon Tn*5*), dyhydroorotate dehydrogenase (from *Saccharomyces cerevisiae*) and puromycin-*N*-acetyltransferase (from *Streptomyces alboniger)*. These markers confer resistance to WR99210, blasticidin S HCl, geneticin/G418, atovaquone and puromycin respectively.

Transfection plasmids are available for both allelic exchange and transgene expression strategies. The development of constructs expressing negative selectable markers such as thymidine kinase and cytosine deaminase/uracil phosphoribosyl transferase has proven valuable. The use of these markers, in conjunction with the positive marker human *dhfr*, reportedly enables the selection of parasites in which the plasmids have integrated via double crossover events. Another approach involves cloning a second selectable marker downstream of the integration site in a manner that would allow the endogenous gene promoter at the site of integration to drive expression

of the marker. These recent approaches aid in selecting for recombination into the desired locus (as compared to recombination into genomic sequences that have homology to noncoding, regulatory elements present elsewhere on the transfection plasmids). Further, this approach should help in selecting for integration events that confer a deleterious growth phenotype relative to episomally transfected or wild-type parasites.

While not covered by this protocol, techniques and plasmids also exist for transient transfection assays in *P. falciparum*, for example to study regulatory elements. These plasmids typically use luciferase or chloroamphenicol acetyl-transferase (CAT) for signal detection.

B. Electroporation of circular plasmid DNA into parasites

Before starting the transfection experiment, label all electroporation cuvettes and tissue culture plates for parasite culture. Set the Bio-Rad Gene pulser to 0.31 kV, 950 μF, and infinity resistance. For each transfection, prepare a 3.5 ml of complete medium containing RBCs at 4% hematocrit in a well of a 6 well plate. The transfection procedure should be finished in about 30 to 60 min from the time the parasites are harvested to the time the transfected parasites are put back into the incubator.

We note that the procedure listed below relies on transfection of parasitized RBC. An alternative approach recently published by Kirk Deitsch and colleagues involves transfecting *P. falciparum* by first electroporating uninfected RBC with plasmid DNA, then adding mature parasite stages and allowing them to invade the plasmid DNA-preloaded RBC. This is an effective means of obtaining relatively high levels of luciferase activity and may well represent an improvement for selectable episomal transfection. Nonetheless, the protocol listed below is reliable and works well.

- For each transfection, transfer 2.5 to 3.0 mL of predominantly ring stage parasite culture into a sterile centrifuge tube and spin down the culture at 1600 rpm for 4 min at room temperature.
- Aspirate the supernatant using a sterile glass pipette.
- Gently resuspend the parasite culture pellet in 3 mL of sterile incomplete Cytomix buffer and spin the culture again at 1600 rpm for 4 min at room temperature. Aspirate the supernatant as described above.
- Add the plasmid DNA (50-100 μg/transfection) to the pelleted cells and bring to a final volume of 450 μL with incomplete Cytomix buffer.
- Transfer the suspension into a Bio-Rad Gene Pulser cuvette (0.2-cm), avoiding any air bubbles.
- Perform the electroporation (using the 0.31 kV, 950 μF conditions described above). Time constants between 7 and 11 milliseconds give good parasite transfection efficiency.
- Immediately after the electroporation, aseptically add 1 mL of fresh complete medium into the Gene Pulser cuvette. Add the medium gently down the side of the cuvette as the RBC are fragile at this point.
- When all the electroporations are completed, transfer the Gene Pulser cuvettes back to the tissue culture hood. Gently pipette the cuvette contents into the prepared 6-well tissue culture plates (one well per transfection) containing 3.5 ml media and 4% hematocrit. Pipette another 1 mL of complete medium into the

cuvette and gently transfer most of culture to the well, leaving behind the lysed and dried cells that accumulate around the top of the cuvette.

- When all the electroporations are finished, place the 6-well tissue culture plate into a modular chamber, gas the chamber with 5% O_2, 5% CO_2, and 90% N_2, and place it back into a 37 ^oC tissue culture incubator.
- One to four hours after electroporation, transfer the cultures into 15 ml conical tubes and centrifuge them at 1600 rpm for 4 min at room temperature. Discard the supernatant.
- Resuspend each parasite pellet in 5 mL of fresh complete medium, put it back into a fresh well of the tissue culture plate, gas, and return it to the incubator.
- The following day, feed each well with 5 mL of complete medium containing the appropriate drug.

C. Maintenance of transfected parasite lines

- On the second day post-transfection, smear the electroporated parasite cultures to determine their parasitemia. A parasitemia of 0.8 to 2.4% at this time point is usually indicative of relatively good transfection efficiency. For most plasmids, this typically means that episomally transfected parasite lines will be observed by microscopy in 2 to 3 weeks. From this day onward, feed parasites with medium containing the appropriate selection drug. To select for human *dhfr* or blasticidin S-deaminase, we recommend using complete medium supplemented with 2.5 nM WR99210 or 2.5 µg/mL of blasticidin S HCl respectively.
- Feed the parasites daily on *Days 3-5.*
- On *Day 6*, smear the lines. There should be no live asexual stage parasites. Large numbers of gametocytes are a sign of excessive stress during the transfection and/or subsequent handling procedures. Reasons for this can include poor parasite health prior to transfection, too much time spent outside of the incubator, or poor RBC condition. Similarly, it is not good to see too many "blebby" RBC (if needed, these RBC can be diluted out by replacing 20 to 30% of the culture with fresh uninfected RBC at this time).
- Resuspend the *Day 6* cultures in 5 mL of complete medium containing the drug and transfer them into a sterile centrifuge tube. Spin the tube at 1,800 rpm for 3 min at room temperature (this centrifugation helps to remove toxic lysis products and dead parasite material). Aspirate and discard the supernatant. Resuspend the pellet in 5 mL of medium containing both drug and an extra 0.1 mL of RBC (stock of 50% hematocrit).
- Skip *Day 7*.
- Feed again on *Day 8*.
- Skip *Day 9*.
- Feed again on *Day 10*. Repeat the smearing, centrifugation, and addition of fresh RBC procedures performed on *Day 6*. There should be minimal parasite debris left with few gametocytes and the RBC should be in good condition.
- Feed every second day (*Days 12, 14* etc.) and smear every 4 days until parasites become microscopically detectable on Giemsa-stained slides. Typically, parasites are first visualized on *Days 14 to 24*. To prevent the parasite cultures from lysing, discard 30 to 40% of the cultures and replace them with fresh RBC every 6 or 7 days (i.e. on Day 14 and Day 21).

- Once parasites have been seen, begin feeding them daily. Freeze the transfected parasite lines as soon as the parasitemia reaches ≥ 2%, with >50% rings. Note the growth rate of the episomally transfected cultures, which grow slower than the parental nontransfected lines.
- Expand and harvest the remaining cultures for genomic DNA extraction from trophozoite mature stage parasites to confirm transfection by plasmid rescue. Subsequently, parasite lines can be maintained in 1-mL volumes for long-term propagation.
- Make frozen parasite stocks every month. It is also convenient to prepare genomic DNA once a month to screen for integration by PCR.
- Stable integrants resulting from homologous recombination and single-site crossover of the plasmid into the genome can often be observed between 45 and 90 days post-transfection. These stable integrant parasites typically grow faster than episomally transfected lines. PCR with primers specific to the vector and the plasmid can be used in combination to efficiently screen for the presence in the bulk culture of parasites that underwent the desired recombination event. PCR positive data can be confirmed by Southern hybridization. Once the desired integration has been detected, the corresponding parasite lines can be cloned by limiting dilution and detected using the Malstat lactate dehydrogenase reagent or by flow cytometry.

References

Crabb BS, Cooke BM, Reeder JC, Waller RF, Caruana SR, Davern KM, Wickham ME, Brown GV, Coppel RL, Cowman AF. 1997. Targeted gene disruption shows that knobs enable malaria-infected red cells to cytoadhere under physiological shear stress. Cell 89(2):287-296.

Crabb BS, Cowman AF. 1996. Characterization of promoters and stable transfection by homologous and nonhomologous recombination in *Plasmodium falciparum*. Proc Natl Acad Sci USA 93(14):7289-7294.

Crabb BS, Triglia T, Waterkeyn JG, Cowman AF. 1997b. Stable transgene expression in *Plasmodium falciparum*. Mol Biochem Parasitol 90(1):131-144.

Deitsch K, Driskill C, Wellems T. 2001. Transformation of malaria parasites by the spontaneous uptake and expression of DNA from human erythrocytes. Nucleic Acids Res 29(3):850-853.

Deitsch KW, Calderwood MS, Wellems TE. 2001. Malaria: cooperative silencing elements in *var* genes. Nature 412(6850):875-876.

de Koning-Ward TF, Waters AP, Crabb BS. 2001. Puromycin-N-acetyltransferase as a selectable marker for use in *Plasmodium falciparum*. Mol Biochem Parasitol 117:155-160.

Duraisingh MT, Triglia T, Cowman AF. 2002. Negative selection of *Plasmodium falciparum* reveals targeted gene deletion by double crossover recombination. Int J Parasitol 32(1):81-89.

Eckland EH, Schneider J, Fidock DA. 2011. Identifying apicoplast targeting antimalarials using high-throughput compatible approaches.
FASEB J 25:3583-3593.

Fidock DA, Nomura T, Talley AK, Cooper RA, Dzekunov SM, Ferdig MT, Ursos LM, Sidhu ABS, Naude B, Deitsch K, et al. 2000. Mutations in the *P. falciparum* digestive vacuole transmembrane protein PfCRT and evidence for their role in chloroquine resistance. Mol Cell 69(4):861-871.

Fidock DA, Nomura T, Wellems TE. 1998. Cycloguanil and its parent compound proguanil demonstrate distinct activities against *Plasmodium falciparum* malaria parasites transformed with human dihydrofolate reductase. Mol Pharmacol 54(6):1140-1147.

Fidock DA, Wellems TE. 1997. Transformation with human dihydrofolate reductase renders malaria parasites insensitive to WR99210 but does not affect the intrinsic activity of proguanil. Proc Natl Acad Sci USA 94(20):10931-10936.

Ganesan SM, Morrisey JM, Ke H, Painter HJ, Laroiya K, Phillips MA, Rathod PK, Mather MW, Vaidya AB. 2011. Yeast dihydroorotate dehydrogenase as a new selectable marker for *Plasmodium falciparum* transfection. MPB 177 (1): 29-34.
Goodyer ID, Taraschi TF. 1997. *Plasmodium falciparum*: a simple, rapid method for detecting parasite clones in microtiter plates. Exp Parasitol 86(2):158-160.
Kadekoppala M, Kline K, Akompong T, Haldar K. 2000. Stable expression of a new chimeric fluorescent reporter in the human malaria parasite *Plasmodium falciparum*. Infect Immun 68(4):2328-2332.
Mamoun CB, Gluzman IY, Goyard S, Beverley SM, Goldberg DE. 1999. A set of independent selectable markers for transfection of the human malaria parasite *Plasmodium falciparum.* Proc Natl Acad Sci USA 96(15):8716-8720.
O'Neill MT, Phuong T, Healer J, Richard D, Cowman AF. 2011. Gene deletion from *Plasmodium falciparum* using FLP and Cre recombinases: Implications for applied site-specific recombination. IJP 41(1):117-123.
Templeton TJ, Kaslow DC, Fidock DA. 2000. Developmental arrest of the human malaria parasite *Plasmodium falciparum* within the mosquito midgut via CTRP gene disruption. Mol Microbiol 36(1):1-9.
Triglia T, Wang P, Sims PF, Hyde JE, Cowman AF. 1998. Allelic exchange at the endogenous genomic locus in *Plasmodium falciparum* proves the role of dihydropteroate synthase in sulfadoxine-resistant malaria. EMBO J 17(14):3807-3815.
Waller RF, Keeling PJ, Donald RG, Striepen B, Handman E, Lang-Unnasch N, Cowman AF, Besra GS, Roos DS, McFadden GI. 1998. Nuclear-encoded proteins target to the plastid in *Toxoplasma gondii* and *Plasmodium falciparum*. Proc Natl Acad Sci USA 95(21):12352-12357.
Wang P, Wang Q, Sims PFG, Hyde JE. 2002. Rapid positive selection of stable integrants following transfection of *Plasmodium falciparum*. MBP 123(1): 1-10.
Wu, Y, Kirkman LA, Wellems TE. 1996. Transformation of *Plasmodium falciparum* malaria parasites by homologous integration of plasmids that confer resistance to pyrimethamine. Proc Natl Acad Sci USA 93(3):1130-1134.
Wu Y, Sifri CD, Lei HH, Su XZ, Wellems TE. 1995. Transfection of *Plasmodium falciparum* within human red blood cells. Proc Natl Acad Sci USA 92(4):973-977.

IV. Site specific genome editing of asexual *Plasmodium falciparum* parasites using zinc-finger nucleases

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This protocol describes fundamental steps to edit the *P. falciparum* genome using engineered zinc-finger nucleases (ZFNs):

A. Customized ZFNs
B. Experimental strategies and plasmid design
C. Drug selection, maintenance and analysis of ZFN-transfected parasite lines

For a detailed transfection protocol please refer to chapter III. **Selectable episomal transfection of asexual stage *Plasmodium falciparum* parasites** by ***Bamini Jayabalasingham*** and ***David A. Fidock***.

IV:A. Customized Zinc-finger nucleases

P. falciparum gene specific ZFNs can be purchased from Sigma-Aldrich. To achieve editing, pairs of zinc-finger proteins with unique specificity for adjacent sequences on either strand of the DNA helix are linked to an endonuclease (FokI) that functions as an obligate heterodimer. ZFNs induce a double-strand break (DSB) that can alter the target by stimulating homologous recombination when a donor template is provided.

To design ZFN binding sites, select 400 to 500bp of sequence encompassing the desired target site of your gene of interest and forward it to Sigma. Although Sigma uses an algorithm to determine the best binding sites it is beneficial to carefully evaluate and select the optimal sequence around the target site. The best results are obtained with ZFNs that bind to GC-rich sequences; therefore it is critical to include stretches with a relatively high GC content in proximity of the desired target site. In general the same rules that apply to *P. falciparum* primer design can be used to evaluate ZFN binding sites. They should be unique in the genome and as GC-rich and diverse as possible. These features are more important than placing the ZFN binding site close to the target site. We have seen editing of single nucleotides up to 1kb up and downstream of the ZFN cut site. Low ZFN activity due to weak or unspecific binging on the other hand results in poor editing efficiency.

Sigma will design ca. 10 ZFN pairs (2 weeks) within the provided 400 to 500 bp and re-confirm the correct sequence before starting synthesis and *in vitro* activity evaluation (6 to 8 weeks). Their *in vitro* data are a good indicator for efficiency in the parasite (see below). The two or three pairs with the highest activity will be shipped.

2A linkage of the ZFNs

The ZFNs are not 2A-linked and will each be delivered in a separate plasmid. ZFN1 (Set 1) and ZFN2 (Set 1) will form a functional pair in which ZFN2 contains the 2A sequence at the 5' end, flanked by the restriction enzymes *BglII* and *AvrII*. This 2A ribosome skip sequence permits expression of both ZFNs as two separate polypeptides whose 2A-linked genes are expressed from a single promoter.

To 2A couple the two ZFNs we use the restriction enzymes *BglII* and *XhoI* to linearize the plasmid with ZFN1 (set 1) and to excise ZFN2 (Set 1). Gel-purify both ZFNs and ligate ZFN2 (ca. 1200bp) into the vector containing ZFN1. The 2A-linked pair (ca. 2500bp) can then be excised with the restriction enzymes *XhoI* and *NheI* and cloned

downstream of a *calmodulin* promoter and upstream of an *hsp86* 3'UTR into the *P. falciparum* pDC2 expression vector. To facilitate separation of the 2A linked ZFNs pair on the gel the vector backbone should simultaneously be digested with *MscI* and *HincII* yielding 3 smaller fragments (1598bp, 661pb and 650bp).

IV:B. Experimental design and plasmid preparation

The design of the construct and the choice of selectable marker can be altered depending on the desired final outcome. Plasmids are most often designed to achieve gene disruption ("knockout") or to exchange polymorphic codon. The following two paragraphs describe fundamental strategies for gene editing in *P. falciparum*.

Exchange polymorphic codons:

To edit distinct nucleotides, we recommend building a single plasmid with the zinc-finger expression cassette, an independent selectable marker (human *dhfr*) and a donor sequence of 1.0 kb to 1.4 kb. The donor sequence should span the ZFN target site and contain the desired point mutation(s). In the standard ZFN expression plasmid (pDC2-h*dhfr*) the restriction sites *AatII* and *BstAPI* are the most suitable sites to introduce the donor sequence. To increase the efficiency of editing in the parasite we recommend mutating the ZF binding site in the donor sequence. The following rules apply: mutate guanines in the primary strand of the target site for each ZFN to cytosines; mutating guanines at the 5' and 3' of each zinc-finger triplet (not to be confused with the amino acid triplet of your target gene) will have more of an effect than the guanines in the center of a triplet, and disrupting GG sequences at the junction between two triplets is the most effective way to disrupt binding. In addition, mutating adenines to thymines at the center of a triplet can also be effective. While the number of possible silent mutations depends on your target sequence, we suggest generating at least two mutations in each binding site. After transfection these mutations can also be used to analyze the bulk culture or screen parasite clones.

Gene disruption:

For knockout constructs a variety of strategies are conceivable depending on the final desired outcome. Independent selectable markers can be flanked by homologous sequences and upon integration disrupt or delete all or part of the endogenous locus. Another approach involves cloning a promoter-less selectable marker downstream of the ZFN target site in a manner that would allow the endogenous gene promoter at the site of integration to drive expression of the marker.

Other subtle modifications of the gene include integration of fluorescent reporters or affinity tags.

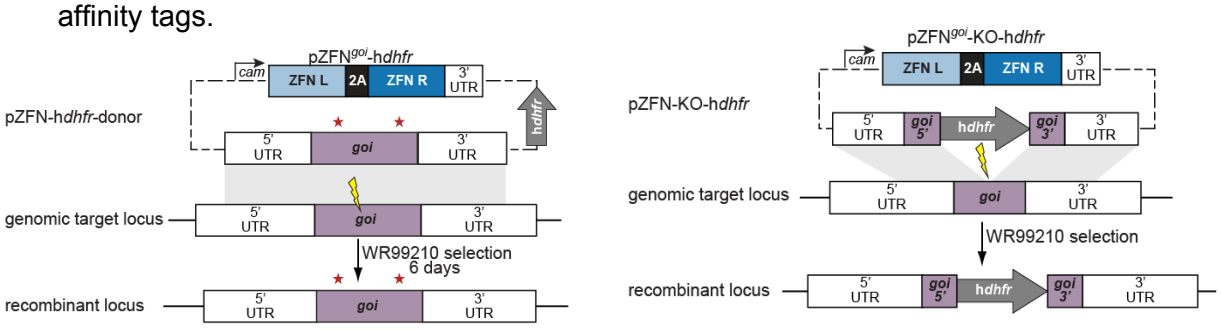

Figure 1. Schematic of gene editing (left panel) and gene disruption strategy (right panel). The plasmids encode 2A-linked left (L) and right (R) ZFNs driven by the

calmodulin promoter as well as a homologous donor sequence carrying the desired point mutations (red stars) on the left or two homologous regions flanking a human *dhfr* (*hdhfr)* cassette on the right. Repair of the ZFN-induced DSB, via homology-directed repair using the donor as template, yields incorporation of the mutations (left panel) and integration of h*dhfr* into the *target* locus (right panel). *goi* (gene of interest), star (mutation), thunderbolt (ZFNs cut site).

For all constructs we recommend a homologous region on either side of the ZFNs binding site of a minimum of 800 bp. If possible design a single plasmid with all the required components: the ZFNs expression cassette, a selectable marker, and the donor construct with the elements designed to modify the locus. Note that one selectable marker on the plasmid is sufficient whether it is independent or only functional when successfully placed under an endogenous promoter of the target site or whether it is designed to be integrated or not. Depending on where the marker is placed, the drug selection protocol after transfection has to be adapted (see below).
For other modifications such as integration of large exogenous sequences and other elements an independent donor plasmid should be build with a second selectable marker. ZFNs can still be expressed from the pDC2-h*dhfr* vector.

For more details on available selectable markers please refer to chapter XI.

Supercoiled plasmid DNA should be prepared as described in XI. Fifty to 100 μg of supercoiled plasmid DNA should be used per transfection. For preparation of the DNA and electroporation of the parasites, please also refer to chapter XI.

IV:C. Drug selection, maintenance and analysis of ZFN transfected parasite lines
For a detailed protocol on preparing parasites for transfection, electroporation and maintenance of parasites please refer to chapter XI.

Appropriate drug selection protocol:
On the second day post-transfection, smear the electroporated parasite cultures to determine their parasitemia. A parasitemia of 0.8 to 2.4% at this time point is usually indicative of relatively good transfection efficiency.
From this day onward, feed parasites with medium containing the appropriate selection drug. If the desired outcome is modification of specific nucleotides and a single plasmid approached was used, drug selection should be applied for 6 days. Feed parasites daily until Day 7 with the drug, then release the pressure and feed the cultures with complete media only. Parasites should be observed by microscope between 15 and 25 days after electroporation. If they are not visible by day 35, discard the culture. Reasons for poor transfection efficiency can include poor parasite health prior to transfection, too much time spent outside of the incubator, or poor RBC condition.

If the experimental design results in integration of a selectable cassette into the final locus then the selection for this marker should be maintained, whereas the selection for the ZFNs expression cassette should not exceed 6 days. We only recommend longer expression of the ZFNs if the activity profile of a particular ZFNs pair is less than 30% compared to the control. These activity data are provided by Sigma.

Maintenance of culture:
Details on how to maintain a healthy culture after transfection are described in chapter XI.

Once parasites have been seen, begin feeding them daily. Freeze the transfected parasite lines as soon as the parasitemia reaches ≥ 2%, with >50% rings.

Expand and harvest the remaining cultures for genomic DNA extraction from trophozoite mature stage parasites to confirm editing by PCR in the bulk culture.

Analysis of ZFN-edited parasites:

PCR with primers specific to the genomic locus and the plasmid can be used in combination to efficiently screen for the presence of parasites in the bulk culture that underwent the desired recombination event. If the desired outcome is editing of distinct nucleotides use two primers outside of the donor sequence, specific to the endogenous locus and sequence analyze the amplified PCR fragment. The electropherograms of the sequencing reaction should show a double peak at the position of the mutated nucleotide, which will indicate that some but not all of the parasites were edited. In our experience the relative height of the two peaks reflects the percentage of successfully edited parasites in the bulk culture.

Once (and only if) the desired modification or integration has been detected in the bulk culture, clones can then be established from the corresponding parasite lines by limiting dilution. Clones are detected using the Malstat lactate dehydrogenase reagent or by flow cytometry.

References

Bibikova, M. et al. Stimulation of homologous recombination through targeted cleavage by chimeric nucleases. Mol. Cell. Biol. 21, 289-97 (2001).

Bibikova, M. et al. Targeted chromosomal cleavage and mutagenesis in *Drosophila* using zinc-finger nucleases. Genetics 161, 1169-75 (2002).

Carroll, D. Genome engineering with zinc-finger nucleases. Genetics 188, 773-82 (2011).

Doyon, Y. et al. Enhancing zinc-finger nuclease activity with improved obligate heterodimeric architectures. Nat. Methods 8, 74-79 (2011).

Doyon, Y. et al. Heritable targeted gene disruption in zebrafish using designed zinc-finger nucleases. Nat. Biotechnol. 26, 702-08 (2008).

Doyon, J.B. et al. Rapid and efficient clathrin-mediated endocytosis revealed in genome-edited mammalian cells. Nat. Cell Biol. 13, 331-37 (2011).

Elliott, B. et al. Gene conversion tracts from double-strand break repair in mammalian cells. Mol. Cell. Biol. 18, 93-101 (1998).

Geurts, A. M. et al. Knockout rats via embryo microinjection of zinc-finger nucleases. Science 325, 433 (2009).

Mimitou, E.P. & Symington, L.S. DNA end resection: many nucleases make light work. DNA Repair 8, 983-95 (2009).

Nkrumah, L. J. et al. Efficient site-specific integration in *Plasmodium falciparum* chromosomes mediated by mycobacteriophage Bxb1 integrase. Nat. Methods 3, 615-21 (2006).

Rouet, P. et al. Introduction of double-strand breaks into the genome of mouse cells by expression of a rare-cutting endonuclease. Mol. Cell. Biol. 14, 8096-8106 (1994).

Straimer, J. et al. Site-specific genome editing in *Plasmodium falciparum* using engineered zinc-finger nucleases. Nat. Methods 9, 993-98 (2012).

Szymczak, A.L. et al. Correction of multi-gene deficiency in vivo using a single 'self-cleaving' 2A peptide-based retroviral vector. Nat. Biotech. 22, 589-94 (2004).

Pabo, C. O., Peisach, E., and Grant, R. A., Design and selection of novel Cys2His2 zinc finger proteins. Annu. Rev. Biochem. 70, 313-40 (2001).

Urnov, F. D. et al., Genome editing with engineered zinc finger nucleases. Nat. Rev. Genetics 11, 636-46 (2010).

V: A Method for Rapid Genetic Integration into *Plasmodium falciparum* Utilizing Mycobacteriophage Bxb1 Integrase

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We have developed a fast and efficient site-specific system of integrative recombination into the *P. falciparum* genome, which is catalyzed by the mycobacteriophage Bxb1 serine integrase. This system has the advantage of providing greater genetic and phenotypic homogeneity with transgenic lines as compared to earlier methods based on episomal replication of plasmids. Herein, we present this methodology.

Integrative recombination constitutes an effective method to target exogenous DNA into specific genomic locations. For bacteriophages, integration into the bacterial host chromosome is typically mediated by tyrosine or serine integrases that catalyze site-specific recombination between phage *attP* and bacterial *attB* sites (1). These phage integrases, which mediate Integration as well as excision reactions, can differ in their requirements for accessory proteins (2). Integration events are highly directional, thus excluding the possibility of spontaneous reversion. Serine integrases of the mycobacteriophage Bxb1 as well as *Streptomyces* sp. phage Φ31 have been extensively used for *in vitro* integration. In particular, the stability of the *attB* x *attP* locus generated by the Φ31 system has been well documented in yeast and bacteria (3, 4).

Mycobacteriophage Bxb1 is a temperate phage that integrates its genome at an *attB* site located within the *Mycobacterium smegmatis groEL1* gene. Recombination occurs between a 48 bp *attP* and a 38 bp *attB* site, each of which contains interrupted inverted repeats that flank a conserved 5'-GT dinucleotide (5). The non-palindromic nature of this central 5'-GT dinucleotide determines the directionality of integration. Unlike the tyrosine integrase systems, serine integrase-based recombination only requires the recombinase to efficiently catalyze the reaction, with no requirement for bacterial host cofactors, divalent cations, or DNA supercoiling (5). These properties make the serine integrase-mediated recombination system an ideal tool for targeted gene delivery in heterologous systems.

We have applied integrase-driven recombination to *P. falciparum*, and find that transiently expressed mycobacteriophage Bxb1 integrase can efficiently catalyze recombination between an *attP*-containing plasmid and an *attB* site integrated into the parasite genome (6).
The integrase-based recombination offers several advantages over episomal-based transgene expression: namely, a higher proportion of fluorescent parasites when the fluorescently-tagged transgene is integrated and a more uniform intensity of fluorescence (**Fig. 1A–C**).
This provides for a more genetically and phenotypically homogeneous expression system as compared to episomal expression, where plasmid copy numbers and transgene phenotypes display higher variability (6, 7). This system has now become a standard tool in our lab to permit studies of transgene expression in phenotypically homogeneous parasite cultures.

Below, we describe the materials and methods of our integrase-based dual-plasmid co-transfection approach, which we now routinely utilize when conducting studies of transgenes expressed as a single-copy from the *P. falciparum* genome.

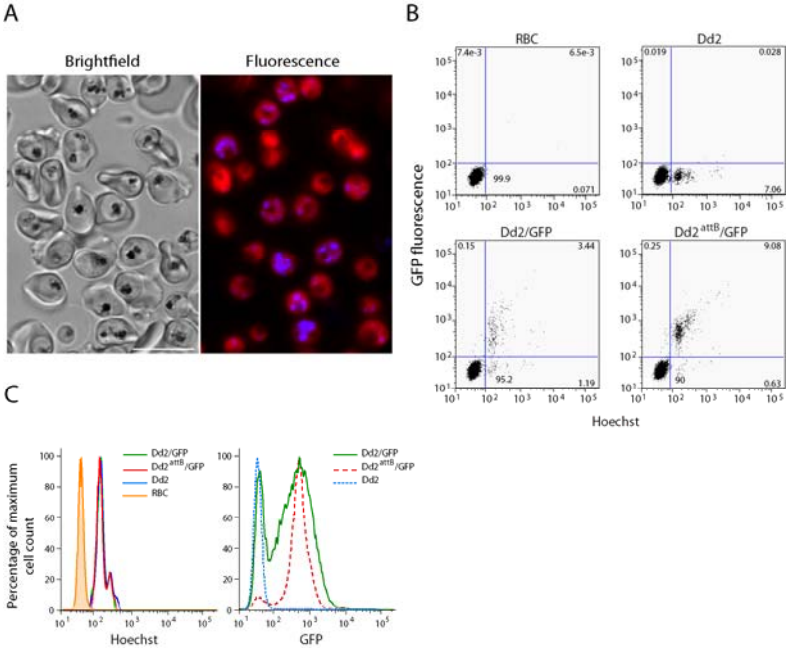

Fig. 1. Generation of homogenous parasites by integrase-mediated *attB* x *attP* recombination. **(A)** Live cell imaging of Dd2^{attB} parasites expressing an mRFP-tagged cytosolic protein (PfVps4) encoded on a plasmid integrated at the *cg6-attB* locus. Bright field (*left panel)* and merged fluorescence images (*right panel*) showing mRFP-PfVps4 (red) and Hoechst 33342-stained nuclei (blue). Prior to imaging, infected RBCs were enriched by magnet-activated cell sorting (MACS, Miltenyi Biotec Inc., Auburn, CA) (12). Bar = 10 µm. (**B**) and (**C**) Flow cytometry analysis of parasites expressing GFP either from a plasmid integrated at the *cg6-attB* locus (Dd2^{attB}/GFP) or from episomes (Dd2/GFP). Parasites were incubated with Hoechst 33342 dye and analyzed at 16-20 hours post-invasion. Cells were gated for nuclear staining to distinguish infected from uninfected RBCs. (**B**) GFP expression profiles show a higher percentage of GFP-expressing parasites in the integrant Dd2^{attB}/GFP population (94%) than in the episomal Dd2/GFP (74%). (**C**) Dd2^{attB}/GFP integrants show a unique peak of GFP expression with negligible variance whereas Dd2 parasites expressing GFP from episomes display multiple peaks with a significantly larger variance. Panels B and C were derived from Figure 4 in (6) with the permission of the Nature Publishing Group.

Transfection and selection of recombinant lines

Materials:

1. Dd2^{attB} and 3D7^{attB} parasite lines harboring an *attB* site integrated at the non-essential *cg6* locus. These lines are available from the Malaria Research and Reference Reagent Resource Center (MR4, Manassas, VA – www.malaria.mr4.org). We note that Dd2^{attB} propagates more rapidly and is our preferred host line.
2. Complete medium (CM) composed of RPMI 1640 with L-glutamine (Invitrogen, Carlsbad, CA), 25 mM N-2-Hydroxyethylpiperazine-N'-2-Ethanesulfonic (HEPES;CalBiochem, San Diego, CA), 50 mg/L hypoxanthine (Sigma-Aldrich, St.

Louis, MO), 0.5% Albumax II (Invitrogen), 0.25% Na_2CO_3 (Sigma-Aldrich) and 0.01 mg/mL gentamicin (Invitrogen). Filter sterilize and store at 4°C.

3. Human Red Blood Cells (RBCs) and Human Sera are obtained from Interstate Blood Bank (Memphis, TN).
4. Plasmids: Integrase-expressing plasmid pINT, which harbors a Neomycin selectable marker that confers G418 resistance; *attP*–containing plasmid pLN-ENR-GFP, which harbors a *pfenr-gfp* expression cassette as well as a *bsd* (blasticidin S-deaminase) selectable marker that confers resistance to blasticidin hydrochloride. pLN-ENR-GFP can be digested with *Afl*II and *Avr*II enzymes (New England Biolabs, Ipswich, MA), to replace the *pfenr-gfp* fusion with the gene of interest. Digestion with *Avr*II and *Bsi*WI (New England Biolabs) can also be performed to place the gene of interest in frame with *gfp*, whose product is positioned at the C-terminus of the corresponding fusion protein. Both the pINT and pLN-ENR-GFP plasmids are available from the Malaria Research and Reference Reagent Resource Center (MR4). Sequence files are available from Genbank (http://www.ncbi.nlm.nih.gov/sites/entrez?db=nucleotide) under the identifiers DQ813653 and DQ813654 for pLN-ENR-GFP and pINT, respectively. Plasmid maps are illustrated in **Fig. 2A, 2B**.
5. Cytomix (1×): 120 mM KCl, 0.2 mM $CaCl_2$, 2 mM EGTA, 10 mM $MgCl_2$, 25 mM HEPES, 5 mM K_2HPO_4, 5 mM KH_2PO_4; pH 7.6. Store at room temperature.
6. Gene-Pulser II (BioRad, Hercules, CA) and 0.2-cm cuvettes (BioRad).
7. Blasticidin HCl (Invitrogen) is dissolved in tissue culture water at 10 mg/mL, sterile-filtered and stored in aliquots at -80*°C*. Add to parasite cultures at a final concentration of 2.5 µg/mL.
8. G418 (Cellgro, Manassas, CA) is dissolved in tissue culture water at 200 mg/mL, sterile-filtered and added to parasite cultures at a final concentration of either 125 µg/mL (Dd2 line) or 250 µg/mL (3D7 line).
9. WR99210 (Jacobus Pharmaceuticals, Princeton, NJ; molecular weight 394.35) is dissolved in CM or tissue culture water at 25.4 mM (10mg/ml), sterile-filtered and stored in aliquots at -80ºC. From this, we prepare working stocks of 25 □M in CM that we keep for up to 4 weeks at 4°C. Add to parasite cultures at a final concentration of 2.5 nM. This antifolate inhibits *Plasmodium* dihydrofolate reductase and selects for recombinant parasites expressing human dihydrofolate reductase (8).

Methods:

Parasites to be transfected are thawed from cryopreserved stocks and placed into culture for 5–7 days to obtain sufficient parasites (see below).

Transfection involves the use of two plasmids, one expressing the gene of interest (which can be prepared by modifying the attP-containing pLN-ENR-GFP plasmid to replace the *pfenr* coding sequence with the desired transgene; **Fig. 2A**) and the other expressing the integrase (such as pINT, **Fig. 2B**). Co-transfection of these plasmids into an *attB* line such as $Dd2^{attB}$ or $3D7^{attB}$ results in transgene integration into the *cg6-attB* site (**Fig. 2C**).

Recombination mediated by mycobacteriophage Bxb1 integrase usually yields a predominant population of integrants, which can be assessed by PCR screening and

plasmid rescue experiments. However, we have also experienced instances where integrative recombination did not occur in a uniform manner but instead led to a mixed population of parasites that either underwent integration ("integrants") or continued to replicate the plasmids episomally ("episomal replicants"). This has been observed with 3D7^{attB}, which propagates more slowly than Dd2^{attB}, leading us to use the latter whenever there is a choice. In such a case of mixed parasite population, it becomes essential to obtain clones by limiting dilution so as to isolate the expected integrants and obtain a genetically homogeneous population. Clones are detected by assaying for parasite-specific lactate dehydrogenase activity (see Section 3.4). The analysis of the recombinant locus is typically performed by Southern Blot to confirm the predicted recombinant locus and evaluate the number of plasmid copies that can integrate in tandem.

Step 1: Prepare the culture for transfection:

1. *P. falciparum* Dd2^{attB} and 3D7^{attB} cultures are propagated in human red blood cells (RBCs) at a 3% hematocrit in CM under reduced oxygen conditions (90% N_2, 5% O_2 and 5% CO_2) as described (8, 9). These lines have been engineered by introduction of an acceptor *attB* site (from *M. smegmatis*) into the non-essential *cg6* locus. This recombinant locus is maintained by continuous selection with 2.5 nM WR99210 (7). The day before transfecting, *P. falciparum* cultures are diluted in freshly drawn, uninfected RBCs to obtain predominantly ring-stage parasites at 5 to 8% parasitemia on the day of transfection.

Step 2: Transfection

Transfection procedures are carried out in Cytomix and the final volume of infected cells and plasmids in the buffer should be 450 µL.

1. Prepare 1 well (of a 6 well plate) for each transfection containing 3.5 ml complete medium and 4% hematocrit.
2. 50 to 100 µg of each plasmid are required per transfection. These have to be equilibrated in Cytomix beforehand, to reach a final volume of 150 µL per transfection. When preparing plasmids, the DNA pellet can be either directly dissolved in 1× Cytomix, or resuspended in sterile water or 1× TE buffer (10 mM Tris•HCl, 1 mM EDTA pH8.0) and diluted 1:1 in 2× Cytomix.
3. On the day of transfection, parasites are first fed with fresh medium and smeared to confirm that the culture contains predominantly ring-stage parasites at greater than 5% parasitemia. Parasite-infected cells are then harvested (2.5 mL per transfection) and centrifuged in sterile conical tubes for 3-4 minutes at 640× *g* at room temperature.
4. Cell pellets are washed once in Cytomix (2.5 mL per transfection) and then resuspended in 300 µL of Cytomix.
5. Plasmid DNA and infected cells are then mixed together and transferred to electroporation cuvettes.
6. Settings for electroporation of infected RBCs are the following: voltage of 0.31 kV and capacitance of 950 µF (when set on maximum capacitance).
7. Immediately after electroporation, 1 mL of complete media is gently and aseptically added to each cuvette. Once all electroporations are completed, the contents of the cuvettes are transferred to the prepared well of a 6-well plates (containing 3.5 mL of

complete media with 4% hematocrit. Cuvettes are then washed with another 1 mL of CM that is added to the well, and the plates are placed at 37º C.

8. Approximately 1 to 4 h after transfection, cultures are transferred to sterile conical tubes and spun for 4 minutes at 450× *g* at room temperature. Pellets are then gently resuspended in 5 mL of complete media and returned to the 6-well plate.

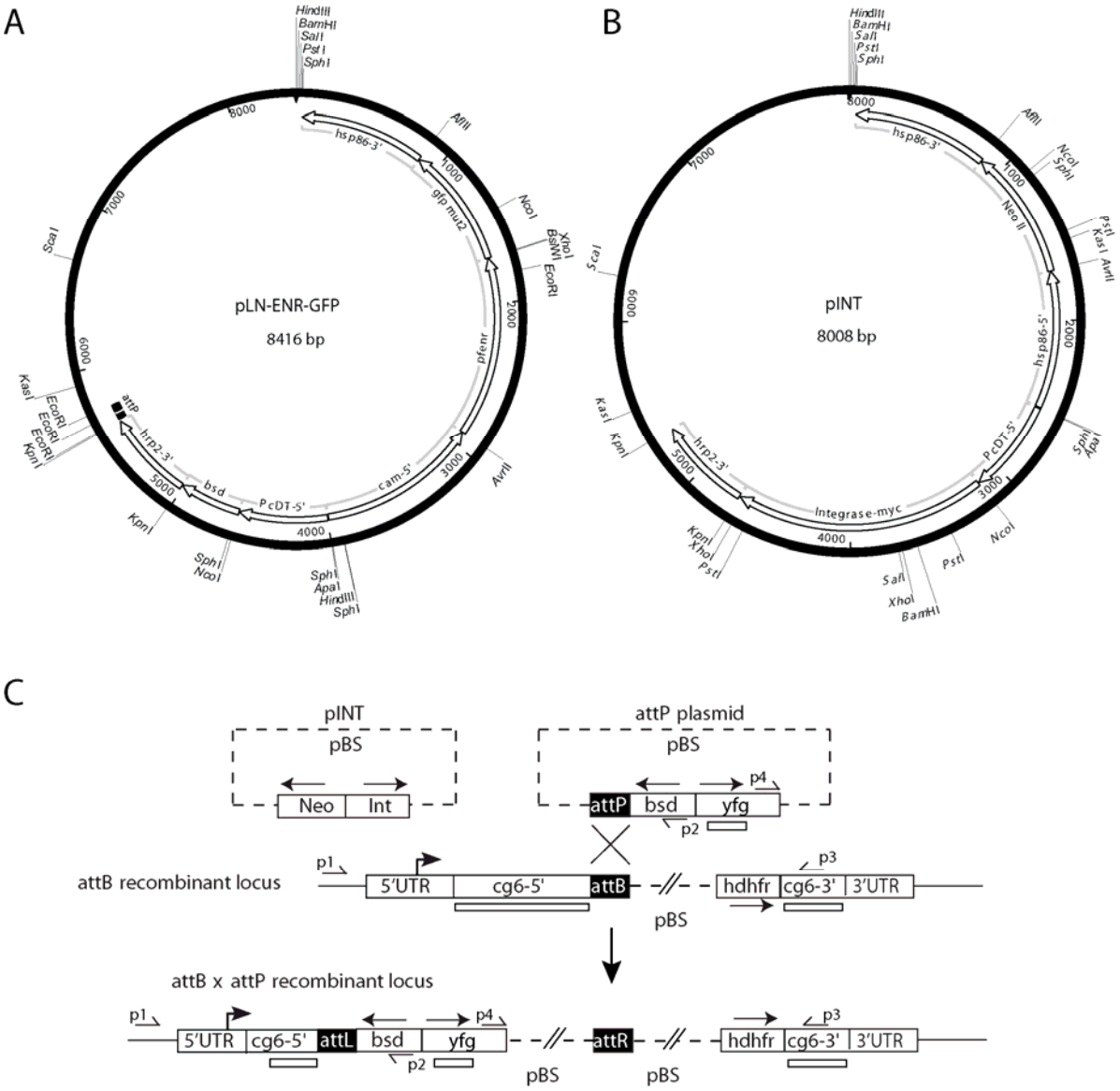

Fig. 2. Dual-plasmid approach for integrase-mediated *attB* × *attP* recombination. (**A**) Map of the *attP*-carrying plasmid pLN-ENR-GFP that can be used as a template for engineering the stable site-specific integration of a gene of interest. Digestion with *Avr*II and *Afl*II can be performed to replace the *enr-gfp* fusion by the gene of interest. Alternatively, digestion with *Avr*II and *Bsi*WI can place the gene of interest in frame with *gfp*. The plasmid carries the blasticidin S-deaminase (*bsd*) selectable marker. (**B**) Map of the integrase-expressing plasmid pINT, which harbors a neomycin selectable marker. The plasmid is usually maintained under G418 selection pressure only for the first 6 days following transfection. (**C**) Schematic representation of the *attB* × *attP* recombination allowing integration of a gene of interest (*yfg*) at the *cg6-attB* locus. Dd2^{attB} or 3D7^{attB} parasites are co-transfected with pINT and an attP-plasmid carrying the gene of interest, and selected with

2.5 µg/mL BSD, 2.5 nM WR99210 (to select for the human dihydrofolate reductase (hdhfr) marker in the locus) and 125 µg/mL or 250 µg/mL G418 (for the $Dd2^{attB}$ and $3D7^{attB}$ parasite lines respectively). Integrase expressed from the pINT plasmid catalyzes integration of the *attP*-plasmid harboring the gene of interest into the *attB* site. PCR reactions using p1+ p2 and p3 + p4 primers as well as Southern Blots analyzing digested-DNA hybridization to *cg6* or *yfg* probes (designated as open boxes), can be performed to confirm integration of the gene of interest at the *cg6-attB* locus. Sequences of the primers for PCR screening are as follows: p1: 5'-GAAAATATTATTACAAAGGGTGAGG, p2: 5'-CTCTTCTACTCTTTCGAATTC, p3: 5'-TTAGCTAATTCGCTTGTAAG, p4 is designed based on the sequence of the gene of interest. pBS refers to the pBluescript backbone. UTR, untranslated region. Panel C was adapted from Figure 3 in (6) with the permission of the Nature Publishing Group.

Step 3: Selection

1. Selection with the agents BSD and G418 (10, 11) starts the day after transfection (i.e. day 1). Cultures are smeared that day to check the post-electroporation parasitemia, which is typically between 1 and 3% (see **Note 1**).
2. Cultures are fed every day for the first 6 days, then every other day, with medium containing the selection agents. Drug-mediated clearance of live parasites is microscopically confirmed on day 6. Integrase-mediated recombination is thought to be a rapid event and it is assumed that maintenance (using G418 drug pressure) of the pINT plasmid expressing the integrase is only required for the first 5 to 6 days. After that time G418 can be removed.
3. On day 6, 100 µL of fresh RBCs (50% hematocrit) is added to each well. On day 12, 30 to 40% of each culture is replaced with fresh RBCs and media by diluting the culture 3:5. This dilution is essential as lysis of aging RBCs can occur after a prolonged period of culture. Transfections are then diluted 3:5 every 6 days and monitored by Giemsa stain every 4–5 days for reemergence of the transfected population. In our experience, $Dd2^{attB}$/ENR-GFP parasites (generated by transfecting the $Dd2^{attB}$ line with the pLN-ENR-GFP plasmid) appeared within 18 days of electroporation. In general, transfected parasites that have undergone *attB* x *attP* recombination are observed 15 to 25 days post-electroporation. In most experiments, the recovered parasites are predominantly integrants, however it is not unusual to detect a subpopulation of parasites containing episomal replicating plasmids in the bulk culture.

Appendix I: Evaluation of proportion of parasites containing integrated plasmid vs. episomal plasmid by plasmid rescue

Plasmid rescue materials:

1. 0.1-cm cuvettes (BioRad).
2. XL1-Blue (or other suitable) Electroporation-Competent Cells (Stratagene, La Jolla, CA).
3. SOC media (Sigma-Aldrich).
4. Luria Broth Base (Becton, Dickinson and Company, Franklin Lakes, NJ) and adequate antibiotic. Both the pINT and the *attP*-containing plasmids possess a carbenicillin resistance marker, which we select with 50 □g/mL carbenicillin disodium (Sigma-Aldrich).

Plasmid rescue methods:

This procedure can be performed to help discriminate between parasites expressing the transgene of interest from either an integrated plasmid or from episomes.

1. extract genomic DNA from transfected parasite culture.
2. 100 ng of genomic DNA is used to transform 50 µL of *Escherichia coli* competent cells (e.g. XL1) by electroporation. Settings for bacteria electroporation are as follows: voltage of 1.8 kV, resistance of 200 Ω and capacitance of 25 µF.
3. Electroporated bacteria are recovered in 500 µL of SOC media (2% bacto-tryptone, 0.5% bacto-yeast extract, 9 mM NaCl, 2.5 mM KCl, 10 mM $MgCl_2$, 20 mM glucose, pH 7.0) at 37ºC for 1 h. 250 µL of recovered bacteria culture are plated on LB/agar with the appropriate antibiotic selectable marker. We use 50 □g/mL carbenicillin, which selects for expression of beta-lactamase by the pLN-ENR-GFP and pINT plasmids and their derivatives. As an alternative, 100 □g/mL ampicillin can also be used, although this tends to produce more resistant satellite colonies upon extended bacterial culture. Quantification of the rescue efficiency provides a reasonably accurate measure of the extent to which the attP-containing plasmids have integrated into the attB site, versus their episomal replication (see **Note 2**).

Appendix II: Detection of integration by PCR

A PCR strategy is generally designed to confirm the generation of *attL* and *attR* flanking sites upon integrase-mediated recombination between the genomic *attB* site and the plasmid *attP* site, as well as the absence of the wild-type locus and of plasmid episomes. Primers used for these PCR screenings are sketched in Fig. 2C (see figure legend for primer sequences).

Appendix III: Notes

1. If the parasitemia is below 1%, it might be necessary to repeat the transfection, as the probability of having transfected parasites is reduced. If the parasitemia is above 3%, cultures have to be closely monitored for the first few days following drug pressure, and culture dilution might be required to prevent parasites dying from overgrowth rather than drug selection.

2. The rescue efficiency, considered as the number of cfu (colony-forming units) per µg of genomic DNA, correlates with the number of episomal replicating plasmids. We generally estimate that integrated plasmids will produce 10^2 cfu/µg DNA (because of instances of integrated plasmids "looping out" of the recombinant locus (7)), whereas episomal replicating plasmids will likely generate 5×10^3 to 10^5 cfu/µg DNA.

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References

1. Groth, A. C., and Calos, M. P. (2004) *J. Mol. Biol.* **335,** 667-78.
2. Ghosh, P., Wasil, L. R., and Hatfull, G. F. (2006) *PLoS Biol.* **4,** e186.
3. Thorpe, H. M., Wilson, S. E., and Smith, M. C. (2000) *Mol. Microbiol.* **38,** 232-41.
4. Thomason, L. C., Calendar, R., and Ow, D. W. (2001) *Mol. Genet. Genomics* **265,** 1031-8.
5. Ghosh, P., Kim, A. I., and Hatfull, G. F. (2003) *Mol. Cell* **12,** 1101-11.
6. Nkrumah, L. J., Muhle, R. A., Moura, P. A., Ghosh, P., Hatfull, G. F., Jacobs, W. R., Jr., and Fidock, D. A. (2006) *Nature Methods* **3,** 615-21.
7. Fidock, D. A., and Wellems, T. E. (1997) *Proc. Natl. Acad. Sci. USA* **94,** 10931-6.

8. Fidock, D. A., Nomura, T., and Wellems, T. E. (1998) *Mol. Pharmacol.* **54,** 1140-7.
9. Trager, W., and Jensen, J. B. (1976) *Science* **193,** 673-5.
10. Mamoun, C. B., Gluzman, I. Y., Goyard, S., Beverley, S. M., and Goldberg, D. E. (1999) *Proc. Natl. Acad. Sci. USA* **96,** 8716-20.
11. Wang, P., Wang, Q., Sims, P. F., and Hyde, J. E. (2002) *Mol. Biochem. Parasitol.* **123,** 1-10.
12. Staalsoe, T., Giha, H. A., Dodoo, D., Theander, T. G., and Hviid, L. (1999) *Cytometry* **35,** 329-36.

VI. Transfection of *Plasmodium falciparum*

(Adapted from: BIOMALPAR/HHMI/WHO Special Programme for Research and Training in Tropical Diseases (TDR) Practical Course)
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V: A. Transfection procedure of *Plasmodium falciparum*

Background

The process that we routinely use for transfection of *P. falciparum* is summarized below. This is the standard procedure for both transient [1, 2] and stable transfection [2-4] although higher initial parasitaemias are used for transient expression. The method shown below uses electroporation of parasite-infected erythrocytes, however, an alternative method of transfection involving spontaneous uptake of plasmid DNA by *P. falciparum* has also been described [5]. Some plasmid constructs lead to the establishment of transfected parasite populations much faster than others. For example, parasites transfected with plasmids designed to express green fluorescent protein (GFP) grow considerably slower than those transfected with other vectors, especially those containing Rep20 sequence (see below). Plasmids should be transfected as undigested circular DNA and will replicate episomally in parasites following transfection and drug selection. Gene targeting by single crossover recombination to disrupt genes or for allelic replacement can be achieved using a drug cycling procedure. Furthermore, gene knockouts using double recombination crossover can be generated using the pHTK plasmid (see below). This transfection plasmid possesses a *thymidine kinase* gene cassette that allows negative selection against the presence of the plasmid backbone and hence selects for parasites that have integrated the positive selectable marker cassette via double crossover recombination. It also greatly shortens the length of time required to derive parasites that have integrated the appropriate portion of the plasmid vector.

Preparation of *P. falciparum* parasites for transfection

Materials and reagents

- Preparation of RBC for transfection: Bags of red blood cells are obtained from the Blood Bank in anticoagulant citrate phosphate dextrose solution. The red blood cells are transferred from the bags to sterile bottles for storage at 4°C and are NOT washed prior to use.
- For 100 mL of RPMI-Hepes, supplement with 5.8 mL of 3.6% NaHCO3 and 10mL of 5% albumax.
  RPMI-Hepes. For a 1 litre solution: RPMI-1640 10.44 g, 25 mM Hepes 5.96 g, 200 µM Hypoxanthine 50 mg, 20 µg/mL Gentamicin 10 mL of a 2 mg/mL stock, H_2O 960 mL, pH 6.72 with 1M NaOH, make up to 1 litre with H_2O, filter sterilise and aliquot, store at 4°C

- 5% albumax: Dissolve 5 g of albumax in 100 mL of RPMI-Hepes at 37 ºC, filter sterilise and store at 4°C.
- CytoMix: 120 mM KCl, 0.15 mM $CaCl_2$, 2 mM EGTA, 5 mM $MgCl_2$, 10 mM K_2HPO_4/KH_2PO_4, pH 7.6, 25 mM Hepes, pH 7.6.
  For 100 mL:
  6 mL 2M KCl
  7.5 µl 2M $CaCl_2$, 1 mL 1M K_2HPO4/KH_2PO_4, pH7.6 (8.66 mL 1M K_2HPO_4 + 1.34 mL 1M KH_2PO_4 = 10 mL 1M phosphate buffer, pH7.6), 10 mL 250 mM Hepes/20 mM, EGTA, pH7.6 with 10M KOH, 500 µl 1M MgCl2, to 90 mL with ddH_20, pH to 7.6 with 0-350 µl 1M KOH, add ddH_20 to 100 mL, Filter-sterilise, store in 3 x 33mL aliquots at 4°C.
- Cytomix stock buffers:
  (a) 10M KOH= 5.61 g/10 mL DDW
  (b) 250 mM Hepes/20 mM EGTA
  5.96 g Hepes (Free acid)
  0.76 g EGTA
  To 80 mL with ddH_20
  pH to 7.6 with 10M KOH(~1.4 mL)
  To 100 mL with ddH_20.
- Pyrimethamine: 200 µM Pyrimethamine (10 mL), add 0.012 g to 5 mL 1% glacial acetic acid (in ddH_20). Have 1% acetic acid at RT. Dilute 200 µl into 10 mL HTPBS (fresh sterile bottle from media each time). Filter-sterilise & store at 4°C. Stable only for 1 month.
- WR99210 (Jacobus Pharmaceuticals): dissolve 8.6 mg WR99210 in 1 mL of DMSO (=20mM). (This may be stored long term at -70 ºC). Dilute 1/1000 in RPMI-Hepes (=20µM). Filter sterilise and store at 4°C. (Stable for 1 month at 4°C)
- Ganciclovir: (Cymevene® for intravenous infusion - Roche). Stock: 51.04 mg in 1 mL ddH_2O (=200mM). Filter sterilize (0.2µm). Store 50 µl aliquots at -70°C. Working solution: dilute stock 1:10 in H_2O (=20mM) (stable for 4 weeks at 4 ºC)
- 0.15% Saponin: Dissolve 0.15g saponin in 100mL RPMI-Hepes, filter sterilise and store at 4°C
- TE buffer: 10 mM Tris-HCl pH 7.5, 1 mM EDTA
- 5% Sorbitol: Dissolve 5g sorbitol in 100mL H_2O, filter sterilise and store at 4°C
- gas mixture of 5% carbon dioxide, 1% oxygen and 94% nitrogen
- GenePulser (BioRad) cuvettes
- GenePulser (BioRad)
- Centrifuge
- 10 cm petri dishes

Procedure

- Synchronise *P. falciparum* parasites at 1-2% ring stages using 5% sorbitol two days before transfection.
- Feed synchronised parasites again 1 day before transfection.
- It is important to use fresh human erythrocytes to ensure that they support growth of parasites during the lengthy initial selection process. The erythrocytes are not washed prior to use
- On the day of transfection parasites should be approximately >5% parasitemia.
- 5 mL of culture (at 4% haematocrit) will be required for each transfection.

Preparation of DNA for transfection

- Prepare plasmid DNA for transfection by ethanol precipitating at least 50 µg of the vector (usually 50-100 µg).
- Allow the pellet to dry for 5 min in a laminar flow hood. Resuspend DNA in 15-30 µl of sterile TE (10 mM Tris-HCl pH 7.5, 1 mM EDTA). It is essential that the DNA be fully dissolved in the buffer before adding further solutions.
- Add 370-385 µl of sterile Cytomix to each plasmid DNA pellet. Cytomix consists of 120 mM KCl, 0.15 mM $CaCl_2$, 2 mM EGTA, 5 mM $MgCl_2$, 10 mM K_2HPO_4/KH_2PO_4, 25 mM Hepes pH 7.6.

Electroporation and plating

- Centrifuge 5 mL of culture/transfection at 1,500 x g for 5 min and remove supernatant.
- Add the Cytomix/plasmid mixture to the parasitised erythrocyte pellet and pipette up and down gently to mix (avoid bubbles!).
- Transfer the parasitised erythrocyte/DNA mixture to a GenePulser (BioRad) cuvette (0.2 cm gap). Electroporate at 0.310 kV and 950 µF. The resulting time constant should be between 7 and 12 msec.
- Immediately add the electroporated sample to a labelled 10 mL petri dish containing 3-4% erythrocytes in complete RPMI/Hepes medium with 10% albumax. Grow parasites at 37°C in a gas mixture of 5% carbon dioxide, 1% oxygen and 94% nitrogen.

Positive drug selection

- 5-8h after transfection change medium and add WR99210 at 2.5-10 nM when using the human *dihydrofolate reductase (dhfr)* gene as the selectable marker (see below for other positive selectable marker).
- At day 2, add WR99210 to 2-10 nM when using the human *dihydrofolate reductase (dhfr)* gene as the selectable marker or 0.2 µM pyrimethamine when using the *Toxoplasma gondii dhfr* gene as the selection system (see other positive selectable markers below).
- Fresh media and the appropriate drug are added to cultures daily for the first 4 days then every 2 days until parasite establishment.
- Smear parasite culture on day 2 post transfection to check for the presence of rings (there should be almost no rings present).
- Add fresh RBC (~100 µl) once a week.
- Parasitised erythrocytes should be detectable in Giemsa stained smears of erythrocytes after 7-75 days (average 21-30 days).
- To select parasites with the plasmid vector integrated by homologous recombination, grow the parasites for 3 weeks without drug selection then reapply drug pressure and continue to culture until parasites (rings) appear in blood smears.

- The parasites should be analysed by Southern blotting (or alternative means) to determine if integration into the relevant gene has been obtained (see details below).
- Continue drug cycling until no death observed after addition of drug.

Negative selection using Thymidine kinase vectors

- For selection of transfected parasites using vectors containing the thymidine kinase gene for negative selection the parasites should be transfected as described above and selected with WR99210.
- Once stable transfected parasites are established on WR99210 add ganciclovir to 20μM to a synchronised culture of 2% rings. (Important: WR99210 selection continues during ganciclovir treatment). During this period there may be substantial parasite death.
- Grow the culture until parasites re-appear and until parasite growth is firmly established.
- Analyse chromosomes and genomic DNA of the parasites using PFGE and Southern blotting to determine if integration into the appropriate gene has occurred in these parasites.

V: B. Monitoring transfectants: genetic analysis

Background

It is particularly important to genetically monitor stable transfectants once a drug-resistant population emerges post-transfection and during the drug cycling process. It is important to do this for three key reasons:

- To ensure that transfected populations do not represent naturally drug-resistant mutants, such as those with a mutation in the endogenous DHFR-TS gene, but are instead transformed with the desired plasmid,
- To determine if the transfected populations possess episomally replicating and/or integrated copies of the transfection plasmid and
- To examine the nature of the integration event (ie, homologous vs non-homologous; single vs double crossover recombination). A combination of three approaches can be used for this analysis:

PCR

This approach can be used for all requirements. However, for a number of reasons we believe that its use is limited and that the technique should be used as a guide only and results have to be confirmed using other techniques. For example, detection of the transfection plasmid by PCR using oligonucleotides specific for a unique sequence (such as a targeting sequence) is confounded by the presence of residual DNA left over from the original transfection. This DNA can be destroyed by pre-digestion of the gDNA with Dpn I, a restriction enzyme with a frequently found recognition sequence that cleaves only methylated (such as that replicated in *E.coli*) and not un-methylated (parasite replicated) DNA, although *Dpn*I digestion is unlikely to be 100% efficient. PCR is particularly useful to detect the presence of homologous integration events using a combination of a plasmid-specific oligonucleotide (not specific to the gene targeting sequence) and one directed to genomic sequence located immediately outside of the gene targeting fragment found in the plasmid. The presence of such a product (which should be sequenced for confirmation) demonstrates that homologous integration has indeed occurred. Using this approach, however, it is not possible to determine the proportion of the parasites that possess integrated forms of the plasmid.

Pulsed-field gel electrophoresis (PFGE).

The separation of *P. falciparum* chromosomes by PFGE followed by Southern blotting is a powerful approach to monitor the genotype of transformants [6, 7]. By hybridizing identical blots with a probe to detect the presence of the transfected plasmid (eg. plasmid backbone or selectable marker gene) and a probe to detect the targeting sequence present in the plasmid, the progress of episomally replicating to integrated plasmid can be followed. While this approach demonstrates plasmid integration and the chromosome into which this has occurred, it does not reveal the specific nature of the integration event. Good examples of the use of PFGE to analyse transfectants is shown in papers by Baldi *et al* [6], Gilberger *et al* [8] and Maier *et al* [9].

During pulsed field electrophoresis (PFGE) macromolecules are subjected to alternately pulsed electric fields and results in the separation of double stranded linear DNA molecules up to 3 megabases, a size range that includes the chromosomes of *P. falciparum*. The resolution of different size DNA molecules can be optimised during PFGE by varying the applied voltage and the pulse time of the alternating electric field.

The separation of *P. falciparum* chromosomes by PFGE can provide a powerful tool in the analysis of transfected parasites in a number of different ways:

- Detection of true transfectants
  Hybridization of plasmid backbone or the selectable marker sequence to southern blots of pulsed field gels allows the differentiation of true transfectants from drug resistant mutants. These probes will not hybridize to DNA from a drug resistant mutant.

- Differentiation between episomal and integrated plasmid
  Circular DNA molecules migrate in an aberrant manner during pulsed field electrophoresis that differs dramatically from the migration of linear molecules of the same molecular weight. Therefore electrophoresis conditions can be chosen that allow easy differentiation between episomal and integrated plasmids following hybridization of plasmid backbone and target sequence to Southern blots of pulsed field gels.

- Chromosomal localisation of integration events
  Hybridization of duplicate Southern blots with either the plasmid backbone or selectable marker sequence and a probe that detects the targeting sequence will reveal whether an integration event has occurred on the correct chromosome. This approach will not reveal the specific nature of the integration event. Successful targeting of the endogenous locus must be confirmed by Southern hybridization of genomic DNA that has been digested with appropriate restriction enzymes.

- Differentiation of integration by single and double crossover recombination
  Transfection plasmids containing a marker for negative selection such as *thymidine kinase* integrate into the genome via double crossover recombination events that integrate the positive selectable marker without the plasmid backbone.Therefore hybridization of duplicate Southern blots of a pulsed field gel with the plasmid backbone and the positive selectable marker sequence will differentiate between integration by single and double crossover recombination. Only the positive selectable marker will hybridize to double crossover integration events and the plasmid backbone will not.

- Molecular karyotype analysis of parent and transfectant
  It can take several months to generate a gene knockout parasite so before any phenotype analysis is carried out it can be worthwhile to confirm that the parent and the knockout have an identical karyotype. Karyotype analysis can be done by ethidium bromide staining chromosomes that have been resolved by PFGE. This will also detect any chromosomal rearrangements that have occurred during the transfection experiment. Further comparison of the genome of the parent and knockout parasites can be done by hybridization of the repetitive probe rep20 to genomic DNA digested with HindIII.

Southern blotting of digested gDNA.

This is the ultimate confirmation of an integration event. To determine if your plasmid has integrated into the intended locus by homologous recombination, gDNA (~1 µg) is digested by appropriate restriction endonucleases, separated by agarose gel electrophoresis and Southern blotted to a transfer membrane. The enzymes to be used should be ones that are intended to reveal a distinct difference in size of the fragments representing wild-type locus, integrated locus and episomal plasmid when the blot is hybridized to a targeting sequence probe. It should be noted that plasmids that integrate by cross-over recombination event sometimes insert a number of head to tail plasmid copies into the locus. If this has occurred a band corresponding to that expected for the episomal plasmid will be observed. Southern blots can be detected using ^{32}P- or Digoxigenin labelled probes.

Materials and reagents

- 10 x Maleic Acid Buffer (500mL):
  58 g Maleic Acid (final conc. 10M), 43.8 g NaCl (final conc. 1.5M), adjust the pH to 7.5 with NaOH (pH changes quickly), autoclave.
- Washing Buffer: 1 x Maleic Acid, 0.3 % Tween 20.
- 10 x Blocking Solution: Dissolve Blocking Reagent 10% (w/v), (bottle 4 of DIG kit, Roche Catalogue # 1 636 090) in 1 x Maleic Acid Buffer at 65°C. Place on stirrer and mix, autoclave. Store at 4°C.
- Detection Buffer: 0.1M Tris HCl, 0.1M NaCl pH 9.5
- CSPD (vial 5) 100 x. Thaw 100 x CSPD (vial 5 from DIG kit) when the kit arrives and make 20 µL aliquots. Freeze these aliquots. Avoid repeated freeze/thaw cycles. A 20 µL aliquot diluted 1:100 with Detection Buffer makes up 2mL (enough for the chemiluminescent detection of a 10 x 10 cm membrane).
- Denharts Hybridization Solution: 6 x SSC, 5 x Denharts,0.1%SDS
- Saponin: Dissolve 0.15 g saponin in 100 mL RPMI-Hepes. Sterilise by filtration through a 0.2 µm filter.

Preparation of *P. falciparum* genomic DNA (A-Phenol/Chloroform precipitation)

Material and reagents

- 3M Na Acetate pH5.0
- 95% and 70% ethanol
- TE-buffer
- 18% SDS
- phenol/chloroform
- chloroform
- alt. DNeasy tissue kit (Qiagen)

Procedure

- Spin culture at 1200 rpm for 5 minutes to pellet parasitised red blood cells and remove supernatant.
- Gently resuspend pellet in 4 volumes of buffer A (1.6 mL).
- Add 1 volume of 18% SDS (0.4 mL) and mix thoroughly then let sit for 2-3 minutes.
- Add 8 volumes of phenol/chloroform (2.4 mL) and mix thoroughly
- Spin at 3500 rpm for 10 minutes.

- Remove aqueous phase into a clean corex tube and ethanol precipitate by adding 1/10 volume of 3M Na Acetate pH5.0 (250 µL) and 2.5 volumes of ethanol (6.5 mL).
- Leave at -20 °C for at least 1 hour, but the preparation could be stored overnight at -20 °C at this stage.
- Pellet DNA by spinning at 10,000 rpm for 10 minutes.
- Drain off the ethanol.
- Dissolve DNA in 600 µl of TE and transfer to an Eppendorf tube.
- Extract twice with phenol/chloroform by adding 600 µL of phenol/chloroform, mixing and spinning at 4,000 rpm for 3 minutes then removing aqueous phase to a clean tube.
- Extract once with chloroform by adding 600 µL of chloroform, mixing and spinning at 4,000 rpm for 3 minutes then removing aqueous phase to a clean tube.
- Ethanol precipitate DNA by adding 50 µL of 3M Na Acetate pH5.0 (250 µl) and 1 mL of ethanol.
- Leave at -20 °C for at least 1 hr, but the preparation could be stored overnight at -20 °C at this stage.
- Pellet DNA by spinning at 10,000 rpm for 10 mins in a microcentrifuge.
- Remove supernatant and wash DNA pellet with 1 mL of 70% ethanol
- Dissolve DNA in 50 µL TE and store at 4 °C.
- Run 2 µL on a 1% agarose gel to check.

alternatively: Preparation of *P. falciparum* genomic DNA with the B-DNeasy tissue kit, or DNeasy tissue kit (Qiagen)

making a digoxigenin-labelled hybridization-probe:
PCR DIG probe synthesis kit Roche Catalogue # 1 636 090
Southern blot digoxigenin

Material and reagents
- 0.125M HCl (5 mL conc. HCl/400 mL DDW)
- 0.5M NaOH/1.5M NaCl
- 0.5M Tris/1.5M NaCl pH8.0
- DDW
- paper and Hybond-N
- 2 x SSC buffer
- UV cross-linker

Equipment
- Hybridization oven
- water-bath

Procedure
- Run digested genomic DNA (1.0-5.0 µg) on a 0.8% agarose gel o/n at 17V.
- Take UV picture of gel with ruler next to it.
- Depurinate DNA in 0.125M HCl (5 mL conc. HCl/400 mL DDW) for 20min.
- Denature DNA in 0.5M NaOH/1.5M NaCl for 30min.

- Neutralize DNA in 0.5M Tris/1.5M NaCl pH 8.0 for 30 min.
- Cut paper and Hybond-N.
- Build up blot, pre-wet Hybond-N and 1st sheet in DDW (evenly), Blot O/N (or at least 4h).
- Rinse the membrane briefly in DDW.
- Cross-link membrane while still damp: place the membrane on Whatman paper soaked with 2 x SSC.
- UV cross-link wet membrane (700 x 100 μJ/cm^2 on cross-linker).
- Rinse membrane briefly in DDW and allow to air dry (membrane can be stored dry at 4°C).
- Pre-hybridization: (do not allow the blot to dry out once you start)
- Calculate T_{hyb}: T_m = 49.82 + 0.41 (% G+C)-600/length of probe in bp, T_{hyb}=T_m-(20 to 25°C), assuming 100% homology.
- Determine how much DIG Easy Hyb will be used:

	Prehyb. -solution	Hyb. -solution
100cm^2 membrane	10 mL	10 mL
Mini-gel (6.5 x 10 cm)	6.5 mL	6.5 mL
Half-gel (15 x 10 cm)	15 mL	15 mL

- Place correct amount of DIG Easy Hyb in tube and place tube in water-bath set at hybridization temperature.
- Transfer membrane in hybridization bag, add pre-warmed DIG Easy hybridization solution, remove air-bubbles, seal bag (enough buffer that it looks slightly puffy) and perform pre-hybridization at hyb temp. for at least 30 min under slight agitation.

Hybridization

- Prepare hyb solution: add appropriate amount of labelled probe (0.5-1 μL) per mL final hyb solution) + 50μl H_2O in Eppendorf tube, heat to 95°C for 5 min, cool quickly in an ice-bath.
- Immediately add denatured probe to a tube containing appropriate amount of pre-warmed DIG Easy hyb solution and mix by inversion.
- The Denharts Hybridization solution (6 x SSC, 5 x Denharts, 0.1% SDS) can also be used with the DIG system. Use with the addition of 10x Blocking Solution (9 parts Denharts Hyb solution to 1 part 10 x Blocking Solution). Using the Denharts Hyb solution can sometimes reduce background with probes that have a high amount of unspecific binding. Add 100μl of boiled salmon sperm DNA per 25 mL prehyb solution. Hybridize at 62°C. Use less of the DIG labelled PCR probe, ~1-3μl/hybridization.
- Pour out pre-hybridization buffer, add immediately hyb solution containing probe to bag, remove air-bubbles and seal bag.
- Immediately add denatured probe to a tube containing appropriate amount of pre-warmed DIG Easy hyb solution and mix by inversion.
- Incubate bag O/N at appropriate hyb temp, agitate blot gently.
- Pour hybridization solution off (store in Falcon tube at -20°C/can be reused 3-5 times).

Washing the membrane

- Wash membrane twice with 2 x SSC at RT for 5 min in a shaking container (make sure membrane does not dry out).

DIG Chemiluminescent Detection

- Equilibrate membrane in 15 mL washing buffer (0.1M maleic acid, 0.15M NaCl buffer, pH7.5, 0.3% Tween-20) for 1 min.
- Incubate membrane in 100 mL 1% blocking solution (2 mL 10% Blocking solution + 18 mL maleic acid buffer (0.1M maleic acid, 0.15M NaCl buffer, pH7.5) at room temperature for 30min.
- Centrifuge anti-Digoxigenin-AP antibody at 10000rpm for 5 min (to remove aggregates), dilute AB 1:10000 in 1% Blocking solution (2µl in 20 mL) and incubate membrane in it at room temp for 30 min.
- Wash membrane in 100 mL washing buffer (0.1M maleic acid, 0.15M NaCl buffer, pH7.5, 0.3% Tween-20) at RT for 15 min.
- Wash membrane in 100 mL washing buffer (0.1M maleic acid, 0.15M NaCl buffer, pH7.5, 0.3% Tween-20) at RT for 15 min.
- Equilibrate membrane in 20 mL detection buffer (100mM Tris-HCl, 100mM NaCl pH 9.5) for 2 min.
- Remove excess liquid from membrane by dripping one corner dry onto a tissue, transfer into hybridization bag, add 2 mL CSPD onto membrane, remove air-bubbles by stroking it with a wet tissue, cover immediately.
- Incubate at RT for 5 min.
- Squeeze out excess liquid, remove air-bubbles, seal bag and put in exposure cassette and place at 37°C for 10 min.
- Expose film (first one for 5 minutes).

Stripping Probe from Membrane

- Rinse the membrane in DDW for 1 minute.
- Wash membrane at 37°C in 0.2M NaOH containing 0.1% SDS. (2 x 15 min).
- Rinse the membrane in 2 x SSC for 5 minutes.
- Store membrane in a hybridization bag at 4°C.

Preparation of *P.falciparum* chromosome blocks

Material and reagents

- LOW MELTING POINT agarose
- Saponin
- lysis buffer 0.5M EDTA, 10mM Tris pH 8.0, 1% sarkosyl, 2 mg/mL proteinase K (proteinase K added fresh just prior to use
- 50 mM EDTA, 10 mM Tris pH 8.0

Procedure

- For best results use a culture containing 6-10% trophozoites
- Chromosome blocks are agarose plugs that contain chromosomal DNA molecules that can be resolved by pulsed field electrophoresis. Standard procedures for DNA preparation do not yield chromosome-sized DNA molecules because high molecular weight DNA is sheared by mechanical forces during

preparation. *P. falciparum* chromosome blocks are prepared by embedding parasites in agarose followed by *in situ* lysis and deproteinisation.

- Chromosome blocks are stable at 4°C for many years.
- before start: 1. Warm PBS to approximately 50°C.
  2. Make up 2% LOW MELTING POINT agarose in PBS and keep at approximately 50°C while you prepare the chromosome blocks.

- Pellet parasitised red blood cells 1200 rpm 5 minutes and discard supernatant.
- Saponin lyse the red blood cells in 1.5 volumes (600 µl) of 0.15% saponin in RPMI-Hepes on ice for 5 minutes. (The volumes in brackets are appropriate for a 10 mL culture)
- Pellet the parasites 2800 rpm for 10 minutes.
- Carefully discard ALL the supernatant.
- Resuspend parasites in approximately 3x the pellet volume (50 µl) of warm PBS.
- Add an equal volume (50 µl) of 2% agarose in PBS and mix.
- Pipette mixture into block cast and allow the agarose blocks to set on ice.
- Push the blocks into lysis buffer 0.5M EDTA, 10mM Tris pH 8.0, 1% sarkosyl, 2 mg/mL proteinase K (proteinase K added fresh just prior to use). Allow approximately 1 mL of lysis buffer for up to 500 µl of blocks.
- Incubate at 37°C for two days.
- Store blocks at 4°C in 50mM EDTA, 10 mM Tris pH 8.0

Running a pulsed field gel

Material and reagents

- 0.5x TBE/1x TAE in MilliQ H_20
- 1% agarose in 0.5x TBE
- 0.5 – 1 µg/mL ethidium bromide solution in H_2O
- BioRad CHEF pulsed field electrophoresis apparatus
- UV transilluminator

Procedure

The following protocol is suitable for a BioRad CHEF pulsed field electrophoresis apparatus. Gels are run at 13°C.

- Set up the gel casting stand with an appropriate comb.
- Prepare 2 litres of 0.5x TBE* in MilliQ H_20.
- Prepare 100 mL of 1% agarose in 0.5x TBE* (make the volume up to 100 mL with H_2O after boiling in a microwave oven to melt the agarose).
- Allow the agarose solution to cool to approximately 60 °C then pour into the casting stand.
- Allow the gel to set at room temperature then carefully remove comb.
- Equilibrate the electrophoresis samples (1mm slices of chromosome blocks) in 0.5x TBE* for 30 minutes at room temperature.
- Load the samples into wells and seal with 1% LMA agarose in 0.5x TBE* (which has been melted and cooled to approximately 50 °C).
- Pour the remainder of the 0.5x TBE* buffer into the tank and turn on cooling system.
- Place gel in the tank.

- Set the appropriate running conditions (see below) and run the gel.
- To visualise the chromosomes, remove the gel into a suitable container and stain in a 0.5 – 1 µg/mL ethidium bromide solution in H_2O or 0.5x TBE for at least 30 mins.
- Photograph the gel on a UV transilluminator.
- DNA fragments larger than 20kb must be cleaved for efficient transfer to hybridization membranes. Prior to transfer, DNA fragments separated by pulsed field electrophoresis are nicked by acid treatment or UV irradiation (5 minutes on a short wavelength UV transilluminator).
- The DNA can then be transferred and hybridized using standard procedures

Comments
*Use 1x TAE for resolution of the four largest chromosomes 11-14.
For resolution of chromosomes 1-5:
1% agarose in 0.5 x TBE, 60 – 120 s pulse, 6 V/cm (200 volts), 24 hour run.
Episomes generally migrate around 35 mm from the wells. These conditions can be useful for detecting the presence or absence of episomes and integration events into loci on chromosomes 1 and 2.
For resolution of chromosomes 1-10:
1% agarose in 0.5 x TBE, 225 sec. pulse, 4.2 V/cm (140 volts), 60 hour run.
Episomes generally migrate in a smeary pattern around 35 mm from the wells although some will migrate at the compression zone about 10 mm from the wells. These conditions are useful for detecting the presence or absence of episomes and integration events into loci on chromosomes 1 –10. However, it can be difficult to differentiate between episomes and integration if the target chromosome co-migrates with the episomes.
In addition, these conditions can be sensitive to different batches of agarose and TBE buffer.
For resolution of chromosomes 11-14:
1% agarose in 1x TAE, 360 – 800 s pulse, 3 V/cm (100 volts), 96 hour run.
A high gel strength agarose such as BioRad Chromosomal Grade Agarose is required.
Episomes generally run off these gels so these conditions are only useful for detecting integration events into loci on chromosomes 11 –14.

V: C. Monitoring transfectants: phenotypic analysis

Background

Prior to embarking on the transfection experiments, you will have already given some thought as to what possible phenotypic changes may result from the genetic manipulation you will make to the parasites. Obviously, phenotypic analyses will depend on which gene you are studying. Below is a method we use to assess whether knockout of putative invasion-related genes results in an altered erythrocyte receptor use [10],[8], [11], [12], [13].

Erythrocyte invasion assay

It is critical that parasite lines are tightly synchronised and growing well before an invasion assay is set up.

The protease enzyme treatments remove different classes of erythrocyte surface proteins, whereas neuraminidase removes specific sialic acid glycans from surface proteins such as the glycophorins. It is advisable to treat parasitised cells at the early ring stage, since these suffer less adversely from the various treatments and washes.

Material and reagents

- sorbitol
- wash buffer
- Neuraminidase
- Trypsin
- Chymotrypsin
- Trypsin/Chymotrypsin inhibitor

Procedure

- Day 1
- Smear cultures and synchronise using 5% sorbitol 2 days before the assay set up. Adjust parasitaemia to 1%.
- Day 3
- On the morning of the assay set up, synchronise parasite stocks with sorbitol and smear cultures. Adjust parasitaemia to 1% and haematocrit to 4% with fresh erythrocytes. From this point on, it is important to take care when removing supernatants, as we want to maintain the same parasitaemia and haematocrit across different parasite lines and subsequent treatments.

ENZYME	Stock Concentration	Final Conc.
Neuraminidase	1U/mL	0.067U/mL
Trypsin	1.7 mg/mL	1 mg/mL
Chymotrypsin	1.7 mg/mL	1 mg/mL
Trypsin/Chymotrypsin inhibitor	1 mg/mL	0.5 mg/mL

- Treat iRBC with enzymes as follows: Centrifuge the entire 10 mL of culture from both dishes in separate tubes at 1200 rpm for 5 mins. For each parasite line, there should be 400 µL RBC, enough for 4 treatments, 100 µL per treatment. Wash cells by resuspending in 10 mL wash buffer. Spin cells at 1200 rpm for 5 mins and remove supernatant. Add 4 mL wash buffer, resuspend pellets and aliquot 1 mL into 4x 1.5 mL tubes, spin and remove supernatant, resuspend cells gently in 200 µL enzyme solutions comprising:

    1. 200 µL wash buffer,
    2. 20 µL neuraminidase, 180 µL wash buffer
    3. 180 µL trypsin, 20 µL buffer
    4. 180 µL chymotrypsin, 20 µL buffer

- Incubate 1 hour at 37°C with gentle shaking. While cells are incubating, label 10 mL tubes with parasite line and treatment. After 1 hour, resuspend trypsin and chymotrypsin-treated cells in 100 µL inhibitor solution. Incubate at 37°C for 10 mins. Resuspend all cells and transfer to labelled 10 mL tubes for washing steps. Add 5 mL wash buffer (WASH 1). Spin down and remove medium (WASH 2). Resuspend again in 5 mL medium, spin and remove medium (WASH 3). Resuspend to exactly 2.5 mL COMPLETE medium.
- Label a sterile 96-well U-bottom plate, leaving outside wells free, these will be filled with medium to prevent drying out of assay. Set up each parasite line in columns, with triplicate wells for each treatment (4 rows). There will be a total of 12 wells for each parasite line, each with 4 treatments: no enzyme, neuraminidase, chymotrypsin and trypsin. In addition set up a couple of control wells with untreated W2mef parasites. Place plates back in incubator until day 5.
- <u>Day 5</u>
- Smear control wells, if parasites have reinvaded (rings), smear all wells, Giemsa stain slides and count invasion events. Percentage invasion of each parasite line into each population of rbc should be calculated by counting invasion events per 1000 erythrocytes. Compare parental and mutant parasite lines for a switch in receptor usage.

V: D. Vector construction

Background

Construction of appropriate vectors for stable or transient transfection using some of the available plasmids can be problematic due to instability and poor growth in *E. coli*. The main reason for this instability appears to be the high AT composition of the genes and in particular the extragenic region that can be >90% AT. Interestingly, once a construct is obtained it usually remains stable henceforth. Therefore, the problems encountered can usually be overcome by testing a number of *E. coli* strains with different genetic backgrounds to identify one that provides a stable vector. The *E. coli* strains PMC103 and XL10-Gold have proven to be very useful for this problem but can provide poor yields of the plasmid.

Subcloning of fragments into the various transfection vectors such as pHH1, pHH2, pHTK and pCC can be very inefficient and it may be necessary to screen large numbers of *E. coli* colonies to identify those that contain the correct plasmid in an un-rearranged state. This can more easily be achieved by using screening by polymerase chain reaction (PCR), picking a portion of each colony directly into the PCR reaction mixture. This facilitates screening of large numbers of colonies to identify those that have the appropriate structure required.

Common *P. falciparum* transfection plasmids

Stable transfection of *P. falciparum* has used primarily two types of vectors containing either *Toxoplasma gondii dhfr* [2, 3] or human *dhfr* [4] as the gene for selection of transfected parasites. More recently, other genes such as blasticidin, neomycin and puromycin resistance genes have been used successfully for selection of *P. falciparum* transfectants [14, 15]. The structure of some commonly used vectors are shown in the Appendix and are described below.

pHH1

- The pHH1 vector [16], its parent vector pHC1 and derivatives [17, 18] have been useful for gene targeting and for transgene expression to analyse protein trafficking, merozoite invasion and drug resistance. This vector allows integration of the plasmid into the genome of *P.falciparum* by single-crossover recombination. Although very useful, this strategy has a major drawback in that it does not allow selection of gene disruptions that are not lethal but are deleterious to parasite growth. This is because of the persistence of episomal plasmid in some parasites despite growth of transfectants in the absence of drug selection. Reapplication of drug pressure selects for parasites that have the plasmid integrated but also for those that contain the plasmid as an episome. If the parasites containing the integrated form of the plasmid grow more slowly they will be lost in the parasite population and parasites with episomal copies of plasmid will predominate.

pTK vectors

- To overcome the problem of persisting episomal plasmid in transfected *P. falciparum* we developed a new vector (pHTK and derivatives) that utilises the *thymidine kinase* gene to negatively select against its maintenance [19]. This has been very successful as it allows disruption of genes not previously obtained using pHH1 and also significantly decreases the length of time required to select

the *P. falciparum* parasites that have integrated the plasmid. Importantly, this vector allows selection of parasites that have integrated a region of the transfection plasmid by double crossover recombination. This is an important advance for reasons described above but also allows more defined deletions and mutations in the *P. falciparum* genome and will also facilitate the production of double mutations and knockouts into the genome.

pCC vectors

- pCC vectors allow - like the pTK vector - the integration via double crossover recombination [20]. Instead of the *thymidine kinase* gene they use *cytosine deaminase* gene as a negative selectable marker. This selection is more stringent allowing a faster and more reliable selection procedure. In addition the pCC vector series is modular making the exchange of the positive selectable marker cassettes easier (see below) and therefore are used for subsequent 'knockouts' of different genes in the same parasite line.

pHH2

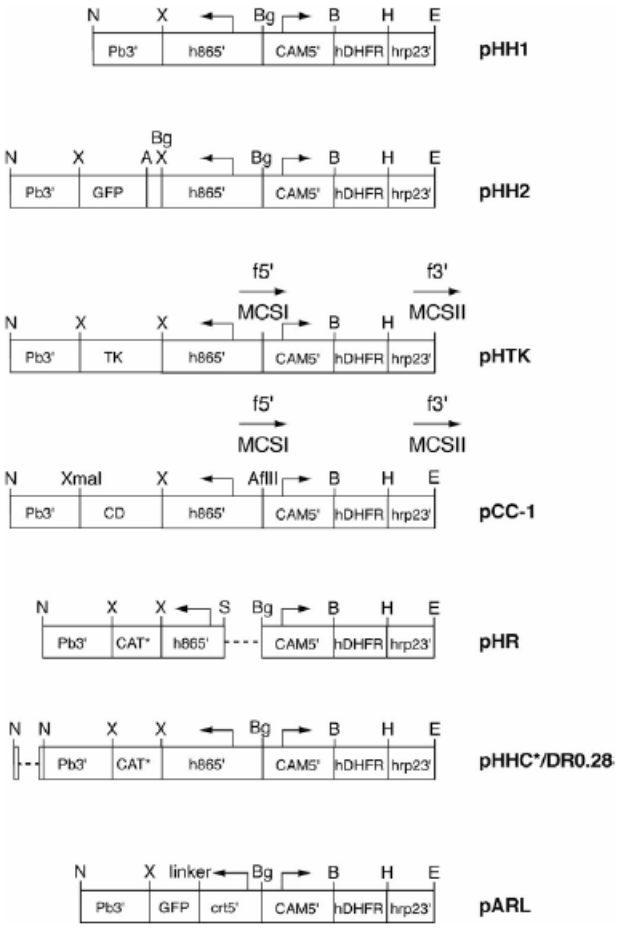
- The use of GFP tagged proteins has been an important application to follow the trafficking pathway of proteins in live *P. falciparum*-infected erythrocytes [18, 21]. The transfection vector pHH2 allows cloning of sequences into a gene cassette to obtain expression of proteins to GFP. This vector uses the promoter from the *hsp86* gene, which allows a broad expression of the GFP in *Plasmodium* blood stages.

pARL-1a

- This vector is mainly used for expression of GFP-tagged proteins. It uses a tail-to-head orientation of the expression cassettes to avoid the bi-directional influence of the *cam* promoter on the expression of the gene of interest. Additional restriction sites facilitate cloning. In contrast to the *hsp*86 promoter driven expression in pHH2, expression in this vector is driven by the *P. falciparum crt* promoter. The distinct *crt* promoter activity has been successful in avoiding cytotoxic levels of GFP expression. For this application the GFP expression cassette of the pHH2 vector is transferred into the *Xho*I site of the pARL-1a vector. The *crt* promoter element can be exchanged to modify the expression profile of the gene of interest.

Rep20 plasmids

- It has recently been shown that the inclusion in transfection plasmids of stretches of the *P. falciparum* subtelomeric repeat sequence Rep20 confers improved plasmid maintenance in transfected parasites [22]. This occurs because Rep20 sequence allows transfected plasmids to tether to *P. falciparum* chromosomes and as a result plasmids are segregated efficiently between daughter merozoites. The primary advantage of this for transfection technology is that drug resistant parasite populations are established much more rapidly if Rep20 is included in the transfection plasmid; some 1-2 weeks before the appearance of parasites transfected with control plasmids.



Other positive selectable markers

In addition to the *dhfr* selectable markers described earlier, 3 other positive selectable markers have been successfully used to derive drug-resistant parasite populations. These markers, blasticidin S deaminase (BSD) [14], neomycin phosphotransferase II (NEO) [14] and puromycin-*N*-acetyltransferase (PAC) [15], confer resistance to blasticidin S, geneticin (G418) and puromycin respectively. Although all three selectable markers have been used to derive drug-resistant parasite populations harbouring episomally replicating plasmids, to date only PAC and BSD has been successfully used for gene targeting.

V: E. Analysis of transient transfectants

Transient transfectants

Background:
Because of the low efficiency of *P. falciparum* transfection, highly sensitive reporter systems are required for use in transient transfection. Two such reporter genes, *chloramphenicol acetyl transferase* (*CAT)* and *luciferase* (*LUC*), have been successfully used for promoter analysis following transient transfection. *LUC* is the more versatile system, but requires more elaborate equipment.

Procedure
See methods for "Preparation of *P. falciparum* parasites for transfection", "Preparation of DNA for transfection" and "Electroporation and Plating". These methods have the following exceptions:

- Ethanol precipitate 75 µg of the plasmid pPf86 (firefly luciferase) and 75 µg of pPfrluc (*Renilla* luciferase) in the same tube.
- Allow pellet to dry in a laminar flow hood. Resuspend completely in 30 µl of sterile TE. DNA may be left at 4°C for several hours prior to transfection to facilitate resuspension.
- Add 370 µl of sterile Cytomix to each plasmid DNA pellet.
- 5h after transfection change the medium.
- Fresh media is added to the cultures 24h post-transfection.

Luciferase assays in *P. falciparum*
Background:
Both firefly and *Renilla reniformis* luciferase genes can be expressed in *P. falciparum* and their respective enzyme activities measured in the same parasite sample [25]. When the *R. reniformis* gene is transfected in conjunction with the firefly luciferase gene, one can be used to control transfection efficiency.

Material and reagents

- saponin
- PBS
- 1x Passive Lysis Buffer (PLB) (Promega)
- Promega's Luciferase Assay Reagent (LAR II) (firefly substrate)
- Promega's Stop and Glo Reagent (Renilla substrate)
- assay tubes/96 well plates (for tube luminometers)

Equipment

- microcentrifuge
- plate luminometer

Procedure:
Preparation of lysates for luciferase assays

- At 48h post-transfection, red blood cells are collected by centrifugation at 1500 rpm for 5 min.

- Prepare desired quantity of 1x Passive Lysis Buffer (PLB) (Promega) by diluting the provided 5x solution with distilled water. Leave on ice.
- Add 1.5x pellet volume of saponin and place on ice for 10 min.
- Centrifuge parasites at 2800 rpm for 5 min.
- Remove supernatant and add 5 mL PBS. Re-centrifuge.
- Remove supernatant and add 1 mL PBS. Transfer to small tubes (Eppendorf). Re-centrifuge to pellet parasites.
- Remove supernatant. Add 50 µl 1x PLB to parasite pellet. Mix well by pipetting or vortexing.
- Incubate on ice for 10 min. Keep these lysates on ice.

Luciferase assay

- Prepare Promega's Luciferase Assay Reagent (LAR II) (firefly substrate) according to the instructions. The efficiency of this reagent decreases with freeze-thaw cycles. It is therefore suggested to prepare all of the provided amount at once and freeze in 1mL aliquots at –70°C. For subsequent assays, these aliquots can be thawed at room temperature.
- Prepare desired amount of Promega's Stop and Glo Reagent (Renilla substrate). 100 µl is required for each sample.
- Thaw the dilution solution in a room temperature waterbath. *Renilla* substrate (Stop and Glo) is provided as a 50x solution. Dilute required amount in appropriate amount of Stop and Glo dilution solution.
- Once prepared these substrates must be kept at room temperature and protected from light.
- For each sample 20 µl of parasite lysate is aliquotted into assay tubes (for tube luminometers) or 96 well plates (for plate luminometers). Follow manufacturer's instructions for operating protocols.
- Add 100 µl LARII. Luminescence is measured for 24-45s.
- Add 100 µl of Stop and Glo and read luminescence for 24-45s. Addition of the *Renilla* reagent quenches the firefly luminescence and simultaneously activates *R. reniformis* activity.
- Normalise the level of firefly luciferase activity to that of *R. reniformis* luciferase activity.

V: F. Inducible gene expression system for *P. falciparum*

Background:
Manipulations of *P. falciparum* blood-stage parasites that have even slight deleterious effects on growth rates are very difficult to derive with the current transfection methods. In order to examine the effect of deleting 'essential' genes or expressing 'dominant-negative' transgenes a conditional mutagenesis system is required in *P. falciparum*. In collaboration with Dominique Soldati's laboratory (Geneva), we have recently developed such a system [26]. This system, which is based on that developed in the related apicomplexan parasite *T. gondii* [27], allows the efficient switching on and off of transgenes using the tetracycline analogue anhydrotetracycline (ATc), in blood-stage *P. falciparum* parasites. The system involves expression of a tetracycline repressor (TetR) protein fused at its C-terminus to different *T. gondii* transactivators (termed TATi1 and TATi2). When bound to tetracycline operator sequences (TetO) placed upstream of minimal (silent) promoters [2] the transactivators facilitate transcription in a manner that is rapidly and efficiently reversed by the addition of ATc. ATc is not toxic for parasites at the levels used, indeed the GPI-anchored GFP transgenic line was generated by 3 weeks culture in the continual presence of ATc. Although much can be done with the system as it stands now in this proposal, we intend to improve the system's useability and adapt it to investigating protein function.
In this course we will induceably express two proteins tagged with GFP from the constructs pTGFP-GPI and pTGFPM19 (Figure 1). The pTGFP-GPI fusion protein contains an endoplasmic reticulum (ER) signal sequence followed by GFP and is terminated with a signal directing the attachment of a glycosylphosphatidylinositol (GPI) anchor (Figure 1A). This protein should traffick to the surface of the *P. falciparum* schizonts/merozoites and remain attached to the outside of the plasma membrane. The second fusion pTGFPM19, is the same as the first except that C-terminal fragment of merozite protein 1 (MSP-1_{19}) has been inserted in frame, in between the GFP and the GPI-attachment signal (Figure 1B).

Procedure:
Tet-inducible GFP expression in *P. falciparum*

- Day1
- starting material is *P. falciparum* blood stage parasites transfected with pTGFP-GPI and pTGFPM19. These parasites should have been grown in the presence of 2.5 nm WR99210 (to select for the maintenance of the plasmids) and 0.5 µg/mL ATc (to keep the expression of the GFP fusion protein off).
- To turn expression of the GFP fusion protein on, the ATc must be removed. This is done by first harvesting the parasite culture and transferring it to a 10 mL tube. The red blood cells infected with ring-stage parasites (iRBC) are then pelleted by spinning the culture at 1500g for 5 mins. To simultaneously synchronize the parasites and remove the ATc, the culture media is removed and replaced with 5 pellet volumes of 5% sorbitol (5% sorbitol in water). The iRBC are resuspended and incubated for 5 mins at 37°C. The iRBC are then pelleted as above and rinsed in 5 volumes of culture media. Finally the iRBC are resuspended in 10 mL of media and 1.25 µL of 20 µM WR99210 is added before the culture is returned to a 10 mL Petri dish.

- Days 2 and 3
- It takes some time for the ATc to diffuse out of the RBC and for its concentration to drop sufficiently so that the parasites can express the GFP fusion protein. For this reason it takes 3 days for the expression of the GFP fusion protein to reach a maximum. To facilitate maximum expression it is important to change the parasite's media each day.
- Day 4
- When the parasites have become schizonts for a second time since having the ATc removed (approx. 3 days later) it is time to look at them under the microscope. Remove 1 mL of iRBC resuspended in their culture media and add 2-5 µl DAPI (50µg/mL) to stain the parasite nuclei.
- Spin parasites at 1500g for 5 mins and remove 400 µl media leaving 100 µL behind in which the iRBC should be resuspended. Drop 5 µl of iRBC onto a microscope slide and gently place a 22 x 64 mm coverslip on top of the cells. Allow the cells to spread out and place under the microscope.

Figure 1A. pTGFP-GPI

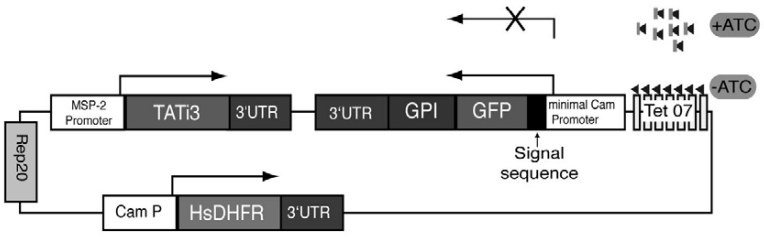
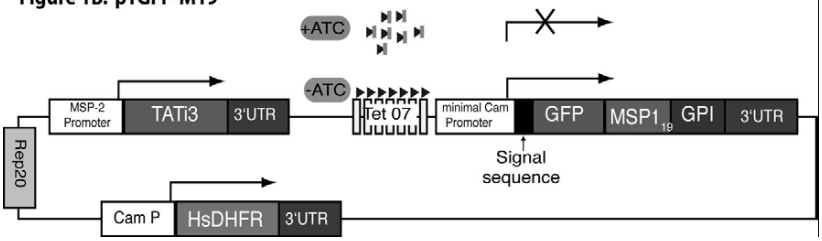


Figure 1B. pTGFP-M19



VII. High-efficiency protocol for *piggyBac* transfection (96-well plate method)

by ***Steven P Maher[1], Min Zhang[1], Bharath, Balu[1, 2] and John H Adams[1]***
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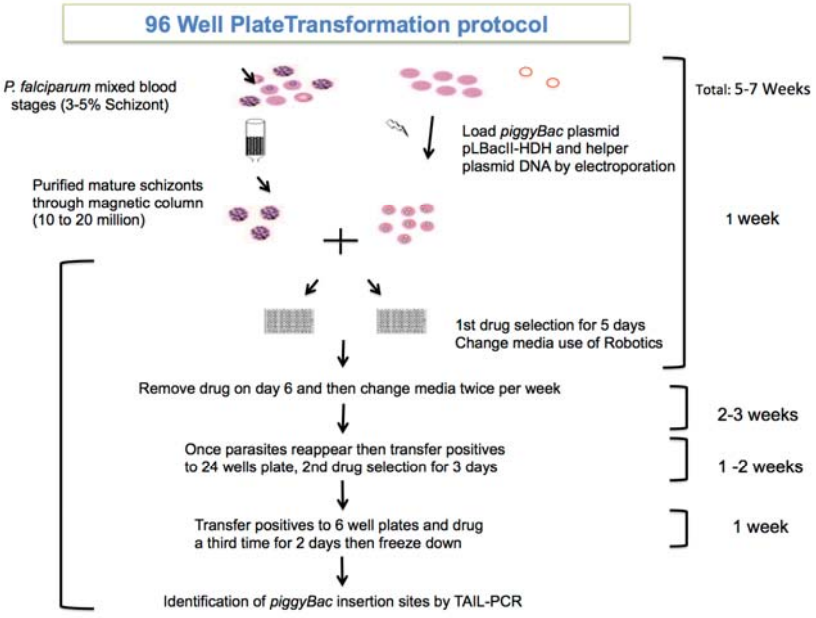
Applications: knocking out and inserting genes into the*Plasmodium falciparum* genome. This protocol is designed to generate single insertion clones of *P. falciparum*using a transposon plasmid (containingselection marker human *dhfr*) and a transposase-expressing helper plasmid. The transposase randomly targets TTAA sites in the genome and active transposition is validated by duplication of the TTAA atthe integrated transposon (Balu et al., 2005, 2009; Cary et al., 1989; Fraser et al., 1995).

The following procedures are split into five parts. The steps have been maximized for high throughput gene knockout production.

1. Preparing material and reagents, plasmid DNA and parasite culture.
2. Loading RBC with plasmid DNA by electroporation.
3. Schizont Purification via Magnetic Column and transformation.
4. Drug selection and maintenance of transfected cultures.
5. Identification of *piggyBac* insertion site by Thermal Asymmetric Interlaced (TAIL) PCR.



A. Preparing material and reagents, plasmid DNA and parasite culture

Materials and reagents

50% washed human red blood cells (O+) free of leukocytes
RPMI 1640 with L-glutamine (Invitrogen, Catalog No. 31800)
Incomplete RPMI medium (RPMI):
1 packet RPMI 1640
5.95 g HEPES (sigma H4034)
50mg Hypoxanthine (sigma H9636)
1L Distilled DI water (Fisher MT25055LB)
Complete RPMI (CM):
25mL 10% Albumax(II, Invitrogen)
15mL 7.5% Sodium Bicarbonate(Invitrogen)
1mL Gentamycin
459 mLIncomplete RPMI
50:50 RPMI:
25mL Human sera (AB+)
12.5 mL 10% Albumax
15mL Sodium Bicarbonate(Invitrogen)
1mL Gentamycin
Add Incomplete RPMI to 500 mL
WR99210:
Make 20mM stock in DMSO and then dilute 1:1000 in RPMI as 20 µM working solution
Cytomix:
6 mL 2M KCl
7.5 µl 2M CaCl2
500 µL 1M MgCl2
1 mL 1M K2HPO4/KH2PO4
10 mL 250 mM HEPES/20 mM EGTA, pH7.6 with 10M KOH
Add distilled water to 100 mL, filter sterilise, store at 4°C
TE buffer
Magnetic large Column (MiltenyiBiotecCS column 130-041-305)
Plasmid Gigaprep kit (Qiagen)
Sterile 96-well, 24-well tissue culture plates
Sterile glass pipettes and other tissue culture plastic ware
Electroporation cuvettes (0.2-cm gap, Bio-Rad)
Storage tubes with barcode (Matrix 0.5 mL 2D screw tubes, Thermo scientific)

Equipment

Gene PulserXcell with CE Module electroporator (Bio-Rad)
Incubator (37°C gas mixture of 5% O2, 5% CO2, and 90% N2)
Biomek 3000 laboratory automation workstation (the “robot”)
Compound Microscope

Plasmid DNA preparation

- Complete a Giga prep for *piggyBac*plasmid and helper plasmid DNA to be used. Dissolve the DNA in 1 mLTE buffer at a concentration around 2 mg/mL in an eppendorf tube
- Before transfection, take 1200 µg piggyBac plasmid DNAand 600 µg helper plasmid DNA add 0.1X volume 3M NaOAc, mix and then add 2.5x volume 100% ethanol.

- Mix and spin for 15min, 12,000g at 4°C.
- Pour off supernatant, wash DNA pellet with 1 mL 70% ethanol.
- Mix well and spin for 10min, 12,000g at 4°C.
- Remove supernatant inside sterile field, let air dry under sterile conditions, and resuspend DNA with 200 µl sterile TE buffer.

Parasite cultures

Obtain at least 40 mL Pf culture with high parasitemiaschizonts (3 to 5% grow in complete RPMI). For each transfection (2x 96-well plate), use 10 to 20 million purified schizonts. The day before transfection, check that the parasite cultures are 3 to 5% healthy ring stage.

B. Loading RBC with plasmid DNA by electroporation

- Need 12 cuvettes, chilled on ice.
- Transfer 6 mL of fresh 50% washed blood into 15 mL tube. Spin down blood, 1,250 g for 3 min, remove supernatant.
- Add 2X Volumes cytomix to 1X volume packed RBC in this manner: For 3 mL blood pellet, use 1 mL cytomix to resuspended plasmid DNA and transfer to blood pellet, then add 5mLmore cytomix to DNA/blood mixture. Mix blood, cytomix, and DNA well using pipette.
- Turn on electroporator (BioRad Gene PulserXCell+CE Module), go to <home>, <exponential protocol>, set V=310, C=950, R=infinity, mm=2, “P” on bottom of screen means it’s ready.
- Load each cuvette with 400µl blood mixture, place cuvettes back on ice for 5min.
- Pulse cuvettes (TC on electroporator should read between 10-15ms), place back on ice for 5min.
- Use sterile extended tip pipettes to transfer loaded RBC’s to a new 15 mL tube.
- Repeat electroporation with same cuvettes one more time (each cuvettes can be used two times).
- Add 2 mL RPMI to 15 mL tube containing loaded RBC’s, mix, centrifuge at 1,250 g for 3 min.
- Remove supernatant, repeat RPMI wash, remove RPMI and then add 2 mLCMto make loaded RBC the volume up to 4 mL, store at 4°C.

C. Schizont Purification via Magnetic Column and transformation

- Spin down culture, 1,250 g for 3min (3-5% parasitemia, high amount of schizonts) and remove supernatant.
- Resuspend parasite pellet in 3 mL RPMI.
- Setup column: attach the 3-way stopcock.
- Fill 10 mL syringe with RPMI and attach to side port of stopcock.
- Fill the column with 10 mLRPMI from bottom using syringe to remove all air from needle and column.
- Wash column with 40 mL RPMI, do not let column run dry. When finished, turn stopcock to stop.
- Load parasite mixture into magnetic column, collecting flow in a 50 mL tube, let run until all of the cultureentersthe filter. When finished, turn stopcock to stop, do not let air into the column.
- Rinse column by adding 2 mLRPMI three times to push through last of RBC’s. Then wash the column with 30 mL RPMI.
- Disengage the column from the magnet and place in a ring stand.
- Elute the column with 30 mL of 50:50 RPMI, collecting effluent in 50 mL tube.

- Spin effluent down, remove RPMI, leaving 1 mL RPMIin the tube and resuspend schizont (black pellet) gently.
- Load10 µl of the schizont resuspension mixture onto a hemacytometer. Put the collected schizonts into the incubator. Count five 4x4 squares.

(# Parasites)(10)(5)(1000)= parasites/mL.

- Pipette 10 million schizonts into basin containing 4 mL plasmid DNA loaded RBC's from part B and 36 mL 50:50 RPMI, mix well but very, very gently.Finish all transfection steps as soon as possible.
- Multichannel pipet 200 µl of culture mix into 2x 96-well plates. Allow plates to incubate overnight.

D. Drug selection and maintenance of transfected cultures

Change media and 1st drug selection

- The next day, make smear of each plate and check for a significant parasitemia of rings (0.3 to 1.0%).
- After parasites reinvade, add WR99210 (final concentration 2.5 nM) to each plate, using robot (Biomek 3000) change media with drug every day for 5 days.
- On day 1, add 2.5 µl of 20 µM WR to 10 mL 50:50 RPMI, change media with drug by robot. 10 mL is amount of media needed per plate when using the robot for media changing/drug application (100 µl media change for each well).
- On day 2-5, add 1.25 µl of 20 µM WR to 10 mL 50:50 RPMI,change media with drug by robot.
- Remove drug on day 6 and then change media twice per week thereafter, adding 125 µl of 50% fresh blood per plate once a week.
- After 14 days post drug, check for parasites twice a week.

2nd Drug Selection and Freezedown

- Once parasites reappear, take note of positive wells from 96-well plates and transfer positives to 24-well plates(1mL media with 5% RBC per well), drug again for 3 days, change media every day.
- The positive wells on 24-well platesthat unaffected by the 2nd drug selection (do not immediately die off)are most likely true insertions. Prepare blood smear 24-well plates every 2 days.
- When parasites appear in 24-well plates (usually takes about 3 days to 1 week), transfer to 6-well plates and drug a third time for 2 days then remove drug.
- When parasite appear in 6-well plates and reach a high parasitemia (>2% rings) they are ready for cryopreservation. Use multi-channel pipet or robot totransfer 4x 200 µl of culture into 96-wellplate, four tips from a multichannel will fit into the 6-well plate to aidthis process.
- Once the freezedown 96-well plate is loaded, spin down and remove supernatant on all wells.
- For the freeze down cultures, prepare a rack by adding the appropriate number of barcode tubes to thebarcode tube holder rack.
- Use the robotic decapper, or if by hand, under sterile condition, decap all barcode tubes. Electronically record the barcode tube numbers.
- Use multichannel pipet to resuspend parasites in freezedown plate in 200 µl freezing media and transfer to barcode tubes.Cap tubes, take barcode rack to LN2 freezer.
- Pellet remaining culture from the 6-wellplates for Tail PCR, genotyping and sequencing, store at -20°C.

E. Identification of *piggyBac* insertion site by Thermal Asymmetric Interlaced (TAIL) PCR

Materials needed

- 3 specific primers:

TAIL PCR 1 primer: Piggybac ITR2 outer primer 5'CATTGACAAGCACGCCTCAC 3'

TAIL PCR 2 primer: Piggybac ITR2 inner primer 5'CTCCAAGCGGCGACTGAG 3'

TAIL PCR 3 primer: Piggybac ITR2 primer 5'AGATGTCCTAAATGCACAGCGAC 3' (the best one for sequencing, though the others work nice as well)

- 4 arbitrary degenerate (AD) primers

1. 5' NTCGA(G/C)T(A/T)T(G/C)G(A/T)GTT
2. 5' NGTCGA(G/C)(A/T)GANA(A/T)GAA
3. 5' (A/T)GTGNAG(A/T)ANCANAGA
4. 5' AG(A/T)GNAG(A/T)ANCA(A/T)AGG

* The specific primers were designed to have Tms (60-65ºC) higher than those (ca. 45°C) of AD.

Method

Each clone will need 4 different reactions. One for each AD primer.The PCR consists of three consecutive reactions. The cycling conditions are as follows:

TAIL PCR 1- Primary (25µl final volume)

12.5 µl of 2X GoTaq buffer (Promega) [includes Mg^{2+}, buffer, dNTPs and Taq DNA polymerase]
2 µl of the pellet of a culture as template (if genomic DNA 25ng)
0.5 µl of first specific primer (stock 10 µM. 200 nM final concentration)
5 µl of AD primer (stock 10 µM. 2 µM final concentration)
5 µl of dH_2O up to 25 µl
1 Cycle 94°C
5 Cycles 94°C (30s), 65ºC (1min), 72°C (2min)
1 Cycle 94°C (30s), 25ºC (2min), ramping to 72°C over 2min, 72°C (2min)
15 Cycles 94°C (30s), 65°C (1min), 72°C (2min)
94°C (30s), 65°C (1min), 72°C (2min)
94°C (30s), 44°C (1min), 72°C (2min)
1 Cycle 72°C (5min)

TAIL PCR 2- Secondary (25µl final volume)

12.5 µl of 2x GoTaq buffer (Promega) [includes Mg^{2+}, buffer, dNTPs & Taq DNA polymerase]
2 µl of the 1/40 dilution of the product of the primary reaction as template (1 µl of the product resuspended in 39 µl of dH2O)
0.5 µl of second specific primer (stock 10 µM. 200 nM final concentration)
5 µl AD primer (stock 10 µM. 2 µM final concentration)
5 µl of dH_2O up to 25 µl
15 Cycles 94°C (30s), 65°C (1min), 72°C (2min)
94°C (30s), 65°C (1min), 72°C (2min)
94°C (30s), 45°C (1min), 72°C (2min)
1 Cycle 72°C (5min)

TAIL PCR 3- Tertiary (50µl final volume)

50µl final volume
25µl of 2x GoTaq buffer (Promega) [includes Mg^{2+}, buffer, dNTPs and Taq DNA polymerase]

4µl of the 1/10 dilution of the product of the secondary reaction as template (1 µl of the product resuspended in 9 µl of distilled water)
1.0µl of third specific primer (stock 10 µM. 200 nM final concentration)
10µl of AD primer (stock 10 µM. 2 µM final concentration)
10µl of dH_2O up to 50µl
40 Cycles 94°C (30s), 65°C (1min), 72°C (2min)
1 Cycle 72°C (5min)
If the TAIL PCR works, following a Southern blot to confirm that it is a clone with single insertion.

References

Balu, B., C. Chauhan, S. P. Maher, D. A. Shoue, J. C. Kissinger, M. J. Fraser, Jr., and J. H. Adams. 2009. piggyBac is an effective tool for functional analysis of the *Plasmodium falciparum* genome. BMC Microbiol 9:83.

Balu, B., D. A. Shoue, M. J. Fraser, Jr., and J. H. Adams. 2005. High-efficiency transformation of *Plasmodium falciparum* by the lepidopteran transposable element piggyBac. Proc Natl Acad Sci U S A 102:16391-6.

Cary, L.C., M. Goebel, B.G. Corsaro, H.G. Wang, E. Rosen, and M.J. Fraser. 1989. Transposon mutagenesis of baculoviruses: analysis of Trichoplusiani transposon IFP2 insertions within the FP-locus of nuclear polyhedrosis viruses. Virology 172: 156-169.

Fidock DA, Wellems TE: Transformation with human dihydrofolate reductase renders malaria parasites insensitive to WR99210 but does not affect the intrinsic activity of proguanil. Proc Natl Acad Sci USA 1997, 94(20):10931-10936.

Fraser, M.J., L. Cary, K. Boonvisudhi, and H.G. Wang. 1995. Assay for movement of Lepidopteran transposon IFP2 in insect cells using a baculovirus genome as a target DNA. Virology 211: 397-407.

Guangliu Yao, Ning Huang, Efficient Amplification of Insert End Sequences from Bacterial Artificial Chromosome Clones by Thermal Asymmetric Interlaced PCR. Plant Molecular Biology Reporter, 1998; 16: 175–181.

Guangliu Yao, Yuanling Chen, High-efficiency thermal asymmetric interlaced PCR for amplification of unknown flanking sequences. BioTechniques 2007, 43:649-656.

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VIII. Transfection of *Plasmodium berghei*

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Background:

In this section, the methods that are typically used to stably transfect P. berghei are described. Details have been extracted from the Leiden Malaria Research Group SharePoint site (www.lumc.nl/con/1040/81028091348221/810281121192556/811070827552556/; see also Databases chapter III,p 462) and more comprehensive information can be found on this site or in the paper Janse et al. (2006) High-efficiency transfection and drug selection of genetically transformed blood stages of the rodent malaria parasite Plasmodium berghei. Nat. Protoc. 1, 346-56.

Since *P. berghei* cannot be readily cultured *in vitro* for more than one cycle, rats or mice infected with *P. berghei* are used as a source of bloodstage parasites for the culture and purification of mature schizonts. Introduction of DNA into these mature stages of *P. berghei* has so far proven to be more successful than into ring and trophozoite stages. The most widely used DNA constructs contain the pyrimethamine resistant form of the *T. gondii* DHFR/TS gene as a selectable marker. This enables transfected parasites, which are injected back into mice, to be selected by treating mice with pyrimethamine. Depending on the desired outcome, the plasmid DNA is either transfected as undigested circular DNA (for episomal replication within the parasite) or as linearised DNA. In the latter scenario, the DNA construct can either be linearised at a unique site located within the target sequence if attempting to integrate plasmid DNA into the parasite genome via a single crossover event, or alternatively, the DNA is digested at the ends of the 5' and 3' target sequence (and preferably also within the vector backbone) so as to remove the plasmid backbone from the rest of the construct in order to drive a double crossover event.

Transfection of P. berghei is usually performed using electroporation with the Amaxa device (Janse et al., 2006, Nat. Protocol. 1, 346-56). The efficiency of transfection for both episomal and targeted integration into the genome ranges between 10-2-10-4 using this device. The high transfection efficiencies obtained with the Amaxa device significantly reduces the time, number of laboratory animals and amount of materials required to generate transfected parasites.

Materials and reagents

- Complete culture medium:
  Culture medium: RPMI1640, with L-glutamine and 25mM HEPES, without $NaHCO_3$ (if no HEPES is present, add 4.95g HEPES per litre culture medium)
  Preparation of medium:
  • dissolve 10.41g RPMI1640 medium in 1 L water (add powder slowly under continuous stirring).
  • add 2 g $NaHCO_3$ (and HEPES if necessary)
  • add 50.000 I.U. Neomycin (stock-solution of 10.000 I.U./mL; Gibco)
  • Sterilise by filtration through a 0.2µm sieve
  • Store at –20^{o}C in 100-200mL bottles

  - Immediately prior to use, Foetal Calf Serum (FCS) is added at a final concentration of 25% (v/v) to give complete culture medium.
- Heparin: Dissolve the content of 1 ampoule heparin (DBL, 5000 I.U per 1 mL) in 25 mL RPMI1640 culture medium (pH 7.2) without fetal calf serum.
- 10 × PBS (Phosphate buffered saline): 0.01 M KH_2PO_4, 1.37 M NaCl, 0.027 M KCl, pH 7.0, Working solution: Dilute the stock 10 × with dimineralised water and adjust the pH to 7.2 with 1 M HCl. Autoclave.
- Nycodenz: Nycodenz powder (Lucron Bioproduct BV) is obtained from Life Technologies. Store at room temperature.
- Nycodenz buffered medium: 5 mM TrisHCL, pH 7.5, 3 mM KCl and 0.3 mM Ca Na_2EDTA.
- Nycodenz stock solution: Dissolve 27.6 g solid Nycodenz in 60 mL buffered medium and make up to 100 mL with that medium (density (20°C) 1.15g/mL). Autoclave and store at 4°C.
- Pyrimethamine-solution in drinking water.
  Dissolve pyrimethamine in DMSO to a final concentration of 7 mg/mL (stock solution). Dilute this stock solution 100 times with tap water and adjust the pH of the water to 3.5-5.0 using 1 M HCl.
- *P. berghei* freezing solution: 30 % glycerol in PBS. Autoclave.
- Parasite lysis buffer: 10 mM Tris pH 8, 0.4 M NaCl, 1 mM EDTA, 1 % SDS.

Procedure:

Preparation of *P.berghei* parasites for transfection

- Infecting donor rats/mice for *P. berghei* transfection (Day 0)
- Day1
- For rats, inject around 1.5×10^7 parasites intraperitoneally (i.p) per rat and these will be ready for harvest around 4 days later (day 4). The parasitemia at harvest should be between 1-4% (no more). 1 rat (approx 5-8 mL heartblood) will be sufficient for up to 12 transfections.
- For mice, inject around 2.5×10^6 parasites i.p into 7-8 new donor mice (or sufficient mice so as to get around 5 mL of heartblood). Parasitemias should be between 1-4 % on day 3. This amount of heartblood will be sufficient for 12 transfections.
- Day 3: In vitro culture of *P. berghei*
- When the parasitemias of the animals reach between 1-4 % heart bleed animals in the afternoon, using a 23 -G needle with attached syringe containing 0.1 mL heparin stock solution. Pool blood from all mice/rat into a 50 mL tube containing 5-10 mL complete culture medium.
- Spin blood at 1500 rpm, 10 min
- Resuspend parasites in complete culture medium such that the haematocrit is between 2-4%, i.e. for 5 mL heartblood of 3% parasitemia culture in around 120 mL complete culture medium. Culture infected RBC in 500 mL erylenmyer or plastic tissue culture flasks. If culturing in flasks without continuous gassing, use around 60-80 mL/flask otherwise can culture 120-180 mL per 500 mL flask.
- Gas flasks and incubate at 37°C overnight. Flasks can either be continuously gassed using an ‘automatic’ continuous gassing system whereby the cultures are continuously gassed throughout the complete culture period using 5% CO_2, 5%

O_2, 90% N_2. Alternatively cultures can be maintained in closed plastic 500 mL culture flasks that have been gassed once for 2 minutes at the beginning of the culture period. (optional – place flasks on a shaker at minimal speed to keep cultures in suspension).

- (Day 4) At around 9.00 am the following day smear parasites by taking out 300 µl of gently resuspended parasites, spinning briefly in 1.5 mL microcentrifuge tube, removing supernatant and resuspending parasites in residual medium. Make smear and stain. Check that the schizonts are nice and healthy.
- *Day 4:* Purification of mature *P. berghei* schizonts
- Split parasite cultures into 50 mL tubes so that have 30-35 mL culture in each. Prepare 55% Nycodenz solution and gently layer 10 mL of this very carefully underneath the suspension. For a culture suspension of 150 mL a total volume of 50 mL of 55% Nycodenz is used (=27.5 mL nycodenz stock solution, 22.5 mL PBS)
- Spin 20-30 min at 1200 rpm in a swing out rotor at room temp, NO BRAKE
- Carefully collect the brown layer containing schizonts (and gametocytes and old trophozoites if present) at the interface with pasteur pipette into new 50mL tube. Uninfected red blood cells will pellet at the bottom of the tube. In general a total volume of about 30-40 mL is collected. Add around 20 mL of culture medium from the top of this nycodenz density gradient to help wash away the Nycodenz.
- Centrifuge parasites at 1500 × g, 8 min to pellet the schizonts.
- Gently remove the supernatant and resuspend pellet volume in 1 mL culture medium for each transfection. Therefore if performing 6 transfections, resuspend in 6 mL of culture medium. Split into microcentrifuge tubes so 1 mL/tube.

Preparation of DNA for *P.berghei* transfection

- Plasmids which are to be maintained episomally in transfected parasites require no digestion and thus 5 µg of DNA can be concentrated for transfection by ethanol precipation. For single and double crossovers, digest DNA with appropriate restriction enzyme(s) overnight to ensure complete digestion and purify the targeting DNA by gel-electroelution or by a gel-purification kit. Ethanol precipitate 5 µg of linearised targeting DNA.
- Allow the pellet to dry and resuspend each DNA construct in 5-10 µl water or TE buffer (10 mM Tris, 1mM EDTA, pH 8).

Electroporation of *P.berghei*

- Add 100 µl of the 'Human T cell Nucleofector™ solution from the Amaxa kit to the resuspended DNA.
- Pellet the schizonts by centrifugation (5s, max speed in microcentrifuge tube). Discard the supernatant.
- Resuspend each parasite pellet gently in the DNA/nucleofector mix.
- Transfer the parasite/DNA/nucleofector mix to a cuvette (0.2 µl) and transfect using the Amaxa gene pulser, protocol U33.
- Add 50 µl complete culture medium to the cuvette immediately after transfection (try to avoid bubbles) and then inject into 1 mouse intravenously using an insulin 27-Gauge needle. Make sure the veins of the mice are swollen first by placing the mice at 37°C for 10 min before electroporation of the parasites.

- Day 5: Drug selection of transfected *P. berghei* parasites
- To select for parasites harbouring transfected DNA provide the animals with drinking water containing pyrimethamine 24-30 hrs after injection of transfected parasites and treat for 4-7 days. Smear the mice every 2nd day from day 5-6 onwards. The parasitemia the day after transfection usually ranges between 0.05-3%. After the first 2 drug treatments a rapid drop in parasitemias occurs to undetectable levels indicating that most of the parasites do not contain the DNA constructs. In successful experiments using the Amaxa electroporator the parasitemia increases to levels of 0.1-5% between days 4 and 7 after transfection. In unsuccessful experiments parasites are often detected between day 13 –15 after the injection of transfected parasites. These parasites are usually non-resistant wildtype parasites that survived the drug treatment protocol.
- At a parasitemia of between 1-5% parasites are collected for storage in liquid nitrogen and for collection of DNA for genotype analysis (see below).

Freezing down of *P.berghei* parasites

- Collect heart blood from 1 mouse with parasitemia around 1-5% using 23-G needle containing around 0.05-0.1 mL heparin solution in the syringe.
- Gently mix blood with an equal volume of sterile freezing solution (30% glycerol made up in PBS).
- Aliquot between 300-500 µl per cryovial. Leave cryovial at 4°C for 5 min then store in liquid nitrogen.

Extracting *P. berghei* genomic DNA from infected mice

- Heart bleed mice displaying parasitemia of between 5-15% using 23-G needle containing around 0.05-0.1 mL heparin solution in the syringe and suspend the blood in 5 mL PBS.

Remove leukocytes from the blood by passing the blood suspension through a Plasmodipur filter (Euro-Diagnostica, www.eurodiagnostica.com) or through CF11 powder. For removal of leukocytes through a CF11 column:

- Add 3 × blood volume of CF11 powder (Whatman) to a column.
- Run through 2 × column of PBS to pack the column.
- Add infected red blood cells (RBC)/PBS mix and collect flow through in a 50 mL tube. Then add a further 2 × column volume and continue to collect flow through. The leukocytes remain bound to the column while the infected RBC will flow through.
- Centrifuge the RBC - 1500 × g, 5-10 min.
- Resuspend the RBC pellet in 1.5 × volume of 0.15% saponin. Leave on ice for 10 min and then centrifuge 1500 × g /10 min.
- Remove supernatant, wash pellet twice with PBS and then store at -20°C until further manipulation for collection of genomic DNA. (Note: this material can also be used to extract RNA or protein).

- FOR DNA EXTRACTION: Resuspend the parasite pellet in 1 mL parasite lysis buffer and mix well
- Add 100 µg RNAse and incubate at 37°C for 30 min. Add 100 µg proteinase K and incubate at 37°C for 45 min.
- Do 3 extractions with phenol/chloroform
- Precipitate the upper aqueous phase (DNA) with 95% ethanol and 1/10 volume sodium acetate pH 5.2 at -20°C for > 1 hr.

- Centrifuge at 13 000 × g for 20 min and then wash the DNA pellet with 70% ethanol
- After drying the pellet at room temp for 5 min, carefully dissolve the DNA pellet in 50-100 µl sterile TE (10 mM Tris, 1 mM EDTA pH 8).

- The transfection plasmid PbGFP$_{CON}$ is used for the stable expression of GFP. This construct contains the pyrimethamine-resistant *T. gondii DHFR-TS* gene for selection of transgenic parasites and an incomplete copy of the *D-SSU-rRNA* as a target region for integration. The vector can be linearised at the unique *Apa*I site for integration. The *GFP* gene is flanked by the *EF-1α a* promoter and the 3' UTR of *P. berghei DHFR-TS.* B: *BamHI*, E: *Eco*RI and H: *Hin*dIII. Extracted from [28].

References

[1] Wu Y, Sifri CD, Lei H-H, Su X-S, Wellems TE. Transfection of *Plasmodium falciparum* within human red blood cells. Proc Natl Acad Sci USA 1995;92:973-7.
[2] Crabb BS, Cowman AF. Characterization of promoters and stable transfection by homologous and nonhomologous recombination in *Plasmodium falciparum*. Proc Natl Acad Sci USA 1996;93:7289-94.
[3] Wu Y, Kirkman LA, Wellems TE. Transformation of *Plasmodium falciparum* malaria parasites by homologous integration of plasmids that confer resistance to pyrimethamine. Proc Natl Acad Sci USA 1996;93:1130-4.
[4] Fidock DA, Wellems TE. Transformation with human dihydrofolate reductase renders malaria parasites insensitive to WR99210 but does not affect the intrinsic activity of proguanil. Proc Natl Acad Sci USA 1997;94:10931-6.
[5] Deitsch K, Driskill C, Wellems T. Transformation of malaria parasites by the spontaneous uptake and expression of DNA from human erythrocytes. Nucleic Acids Res 2001;29:850-3.
[6] Baldi DL, Andrews KT, Waller RS, et al. RAP1 controls rhoptry targeting of RAP2 in the malaria parasite *Plasmodium falciparum*. EMBO J 2000;19:2435-43.
[7] Crabb BS, Cooke BM, Reeder JC, et al. Targeted gene disruption shows that knobs enable malaria-infected red cells to cytoadhere under physiological shear stress. Cell 1997;89:287-96.
[8] Gilberger TW, Thompson JK, Triglia T, et al. A novel erythrocyte binding antigen-175 paralogue from *Plasmodium falciparum* defines a new trypsin-resistant receptor on human erythrocytes. J Biol Chem 2003;278:14480-6.
[9] Maier AG, Duraisingh MT, Reeder JC, et al. *Plasmodium falciparum* erythrocyte invasion through glycophorin C and selection for Gerbich negativity in human populations. Nat Med 2003;9:87-92.
[10] Duraisingh MT, Maier AG, Triglia T, Cowman AF. Erythrocyte-binding antigen 175 mediates invasion in *Plasmodium falciparum* utilizing sialic acid-dependent and -independent pathways. Proc Natl Acad Sci U S A 2003;100:4796-801.
[11] Baum J, Maier AG, Good RT, Simpson KM, Cowman AF. Invasion by *P. falciparum* merozoites suggests a hierarchy of molecular interactions. PLoS Pathog 2005;1:e37.
[12] Triglia T, Duraisingh MT, Good RT, Cowman AF. Reticulocyte-binding protein homologue 1 is required for sialic acid-dependent invasion into human erythrocytes by *Plasmodium falciparum*. Mol Microbiol 2005;55:162-74.

[13] Stubbs J, Simpson KM, Triglia T, et al. Molecular mechanism for switching of *P. falciparum* invasion pathways into human erythrocytes. Science 2005;309:1384-7.
[14] Ben Mamoun C, Gluzman IY, Goyard S, Beverley SM, Goldberg DE. A set of independent selectable markers for transfection of the human malaria parasite *Plasmodium falciparum*. Proc Natl Acad Sci USA 1999;96:8716-20.
[15] de Koning-Ward TF, Waters AP, Crabb BS. Puromycin-N-acetyltransferase as a selectable marker for use in *Plasmodium falciparum*. Mol Biochem Parasitol 2001;117:155-60.
[16] Reed MB, Saliba KJ, Caruana SR, Kirk K, Cowman AF. Pgh1 modulates sensitivity and resistance to multiple antimalarials in *Plasmodium falciparum*. Nature 2000;403:906-9.
[17] Crabb BS, Triglia T, Waterkeyn JG, Cowman AF. Stable transgene expression in *Plasmodium falciparum*. Mol Biochem Parasitol 1997;90:131-44.
[18] Waller RF, Reed MB, Cowman AF, McFadden GI. Protein trafficking to the plastid of *Plasmodium falciparum* is via the secretory pathway. EMBO J 2000;19:1794-802.
[19] Duraisingh MT, Triglia T, Cowman AF. Negative selection of *Plasmodium falciparum* reveals targeted gene deletion by double crossover recombination. Int J Parasitol 2002;32:81-9.
[20] Maier AG, Braks JA, Waters AP, Cowman AF. Negative selection using yeast cytosine deaminase/uracil phosphoribosyl transferase in Plasmodium falciparum for targeted gene deletion by double crossover recombination. Molecular & Biochemical Parasitology 2006;150:118-21.
[21] Wickham ME, Rug M, Ralph SA, et al. Trafficking and assembly of the cytoadherence complex in *Plasmodium falciparum*-infected human erythrocytes. EMBO J 2001;20:1-14.
[22] O'Donnell RA, Freitas-Junior LH, Preiser PR, et al. A genetic screen for improved plasmid segregation reveals a role for Rep20 in the interaction of *Plasmodium falciparum* chromosomes. EMBO J 2002;21:1231-9.
[23] van Dooren GG, Marti M, Tonkin CJ, et al. Development of the endoplasmic reticulum, mitochondrion and apicoplast during the asexual life cycle of *Plasmodium falciparum*. Mol Microbiol 2005;57:405-19.
[24] Tonkin CJ, van Dooren GG, Spurck TP, et al. Localization of organellar proteins in *Plasmodium falciparum* using a novel set of transfection vectors and a new immunofluorescence fixation method. Mol Biochem Parasitol 2004;137:13-21.
[25] Militello KT, Wirth DF. A new reporter gene for transient transfection of *Plasmodium falciparum*. Parasitol Res 2003;89:154-7.
[26] Meissner M, Krejany EO, Gilson PR, et al. Tetracycline analogue-regulated transgene expression in *Plasmodium falciparum* blood-stages using *Toxoplasma gondii* transactivators. Proc Natl Acad Sci U S A 2005;102:2980-5.
[27] Meissner M, Schluter D, Soldati D. Role of *Toxoplasma gondii* myosin A in powering parasite gliding and host cell invasion. Science 2002;298:837-40.
[28] Franke-Fayard B, Trueman H, Ramesar J, et al. A Plasmodium berghei reference line that constitutively expresses GFP at a high level throughout the complete life cycle. Molecular & Biochemical Parasitology 2004;137:23-33.

FIELD BLOOD SAMPLING

I. Finger prick blood sampling

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I:A. Blood collection on filter paper

Equipment

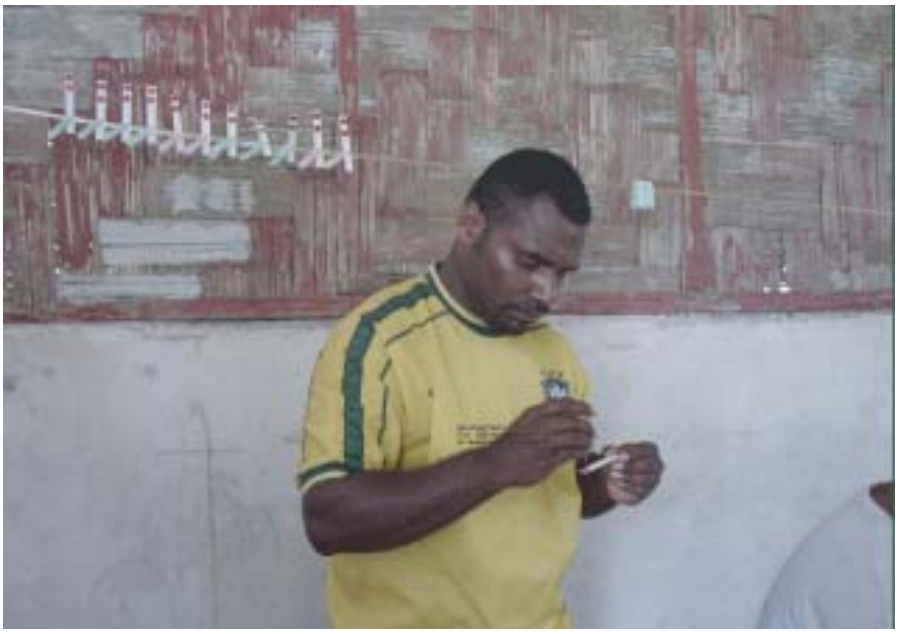
heating block (Techne)

Materials and reagents

heparinizised capillary tubes, 75-μL (Drummond Scientific)
chromatography filter paper, ET31CHR (Whatman).
EDTA tubes (Microtainer, Becton Dickinson)

Procedure

- Draw finger prick blood samples into 1 to 3 heparinized, 75-μL capillary tubes and transfer them to chromatography filter paper.
- Alternatively, allow 1 drop of blood to fall onto a filter paper directly from the finger. The finger must not touch the filter paper (see the photo).
- Soak the drop into the filter paper until the paper is evenly red without spreading to the underlying surface.
- Dry the filter-paper samples completely in air (see the photo) and place them in small plastic bags.
- We can keep the dried filter-paper samples at room temperature for at least several weeks in field conditions before extracting antibodies, human and parasite DNA, and postdose drugs.
- Store the filter-paper samples at −20 °C for long term.



I:B. Blood collection in EDTA tubes

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A better quality and quantity of DNA can be obtained by EDTA tubes than by filter paper.

Materials and reagents

EDTA tubes (Microtainer, Becton Dickinson)

Procedure

- Draw blood samples taken from the finger tip into 2 to 5 heparinized capillary tubes as mentioned above and transfer into an EDTA tube.
- We can keep the EDTA-tube samples at room temperature for at least one week in field conditions before extracting DNA.
- Store the EDTA-tube samples at −80 °C for long term.

II. Serum extraction from filter-paper samples

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Equipment

paper punch
shaker (Vortex)

Materials and reagents

10-mL tubes
PBS containing 0.05% Tween and 0.5% BSA
Pasteur pipettes

Procedure

- Use a paper punch with a diameter of about 6 mm for punching out discs. Be careful to use areas in the middle of the spot where the blood is evenly spread.
- Put the discs in 10-mL tubes.
- Add 500 μL of PBS containing 0.05% Tween and 0.5% BSA.
- Incubate the discs for 2 h at room temperature on a shaker.
- Vortex them for a few seconds.
- Withdraw the liquid with a Pasteur pipette and aliquot it.
- Store at −20 °C until analysis.
- The extract corresponds to a serum dilution of ~1:100. The dilution factor is however dependent on the quality of the filter paper.

Reference: Patrick H Corran, Jackie Cook, Caroline Lynch, Heleen Leendertse, Alphaxard Manjurano, Jamie Griffin, Jonathan Cox, Tarekegn Abeku, Teun Bousema, Azra C Ghani, Chris Drakeley and Eleanor Riley Dried blood spots as a source of anti-malarial antibodies for epidemiological studies. Malaria Journal 2008, 7:195

III. DNA extraction

III: A. DNA extraction from filter-paper samples

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Microscale isolation of *Plasmodium falciparum* or human DNA from filter-paper samples has been conventionally done by brief boiling (Wooden et al. 1992; Uchida et al. 1995), which is sufficient for obtaining short DNA.

Equipment

centrifuge

Materials and reagents

sterile water containing 15 µL of Chelex-100 (Bio-Rad)
HEPES-buffered saline (HBS) containing 0.5% (w/w) saponin (Merck)
1.5-mL tube
QIAamp DNA Mini Kit (Qiagen)
ethanol
DNA elution buffer AE:
0.5 mM EDTA
10 mM Tris–HCL (pH 9.0)

Procedure

- Cut one quarter or half of the dried filter blot, equivalent to 19 or 38 µL of blood, into 3 × 3-mm pieces.
- Heat the pieces in 205 µL of sterile water containing 15 µL of Chelex-100 at 100 °C for 8 min.
- After cooling them on ice for 2 min, collect the supernatant containing DNA (200 µL) by centrifugation and store them at −20 °C.

However, the DNA samples thus obtained show a limited PCR template activity with little amplification of DNA longer than 1 kb. Considering the extremely high A + T content of *P. falciparum* DNA, boiling may cause fragmentation of DNA and would no longer be a method of choice for DNA isolation when a long PCR product covering an entire gene is required. To solve this problem the following alternative method has been developed (Sakihama et al. 2001):

- Cut one quarter or half of the dried filter blot, equivalent to 19 or 38 µL of blood, into 3 × 3-mm pieces.
- Incubate the pieces in 1 mL of HEPES-buffered saline (HBS) containing 0.5% (w/w) saponin at room temperature for 1.5 h in a 1.5-mL tube.
- Wash them twice with 1 mL of HBS.
- Use a QIAamp DNA Mini Kit to isolate DNA remaining on the filter paper, according to the manufacturer's instructions with the following modifications.
- To avoid shearing DNA, minimize vortexing and substitute by repeated inversions.
- Before the addition of ethanol to the extract, thoroughly remove the filter-paper debris by centrifugation at 9,000 × *g* for 5 min.

- Reduce the volume of DNA elution buffer AE to 50 μL.
- Prolong the preincubation before DNA elution to 5 min to maximize the elution recovery.
- Store the eluted DNA at 4 °C until PCR amplification.

References

Sakihama N, Mitamura T, Kaneko A, Horii T, Tanabe K. 2001. Long PCR amplification of *Plasmodium falciparum* DNA extracted from filter paper blots. Exp Parasitol 97(1):50-54.
Uchida JY, Kasahara T, Bobogare A, Saefafia S, Kere N, Kawabata M, Ohta N, Ishii A. 1995. The prevalence of falciparum malaria in the Solomon Islands investigated by a filter paper disk PCR method. Jpn J Parasitol 44(2):119-127.
Wooden J, Gould EE, Paull AT, Sibley CH. 1992. *Plasmodium falciparum*: a simple polymerase chain reaction method differentiating strains. Exp Parasitol. 75(2):207-212.

III:B. DNA extraction from filter-paper discs previously used for serum extraction

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Equipment

Vortex
Heating block
Eppendorf centrifuge

Materials and reagents

Eppendorf tubes
Saponin
PBS
Chelex-100
Distilled water

Procedure

- After serum extraction as described in FIELD BLOOD SAMPLING, section II, transfer the discs to new Eppendorf tubes containing 1 mL of 0.5% saponin in PBS.
- Incubate the tubes at 4 °C overnight.
- Discard the solution.
- Wash the discs with 1 mL PBS at 4 °C for 15-30 min.
- Discard supernatant.
- Transfer the discs into new tubes containing 100 □l of 5% Chelex-100 in water.
- Vortex the tubes for 30 sec.
- Heat at 95 °C for 15 min.
- Vortex the tubes for 30 sec. NOTE: Watch your hand! Caps of tubes are loose and the buffer is hot.
- Centrifuge at 10,000 rpm for 2-3 min.
- Collect the supernatant (using pipette) in new Eppendorf tubes.
- Use 2.5 μl in a 10 μl total PCR reaction mix, store at -20 °C.

III:C. DNA extraction from EDTA-tube samples

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Equipment

water bath
Generation Capture Plate kit (Gentra Systems)

Materials and reagents

whole blood frozen in EDTA tube
EDTA tubes

Procedure

- Use 150 to 375 μL of frozen (−80 °C) whole blood collected in an EDTA tube.
- Thaw it quickly in a 37 °C water bath and keep it on ice until use.
- Use Generation Capture Plate kit according to the manufacturer's instructions.

III:D. Fast methanol-based DNA extraction from blood spots in filter paper.

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Equipment

heating block (Techne)
micropipette, 1000-μL

Materials and reagents

blood spots on filter paper (Whatman 3MM)
new surgical blades
tips
1.5-mL Eppendorf tube
methanol
distilled, sterile water

Procedure

- Cut a blood spot (about 3 × 5 mm) from the filter paper. (Note: Not all filter papers work when making the blood spot collection. Make sure you are using the right one, usually Whatman 3MM.) Do not touch the blood with the fingers when manipulating the filter paper. Use new surgical blades, one for each sample, to avoid contamination. The cuts should be done on a disposable surface, the best choice being small yellow stickers (note pads).
- Use the blade also for transferring the cut-out to a 1.5-mL Eppendorf tube. The filter papers with the remaining blood spots should be protected (e.g., in separate

plastic bags) until a new DNA extraction. After each blood sample, remove 2 or 3 note pad sheets (due to the fact that the blade usually cuts through at least two sheets of the note pad).

- Add 125 μL of methanol (room temperature) and wait 15 min. Make sure that the whole paper-cut is soaked in the methanol. Keep at room temperature until further processed (at least 15 min).
- Remove the methanol with a 1000-μL micropipette (using a new tip for every sample).
- Let the paper dry at room temperature for 15 min with the lid open. Close the lid and rotate the tube gently. If the paper moves freely it is dry, if it sticks even slightly, dry it for 5 to 10 more minutes.
- Add 65 to 75 μL of distilled, sterile water and smash the paper in the water with the micropipette. The water will become slightly red.
- Heat the tube at 95 to 100 ^{o}C in a heating block or a water bath for 15 min.
- Ready for PCR! Use 3 to 10 μL in a 50-μL total reaction volume. (Attention: This extraction is rather dirty. Too much template can give rise to inhibition of polymerase.)

Note: FIELD BLOOD SAMPLING, section III:A for DNA extraction is suitable for long, intact fragments over 700 bp, and is particularly efficient for fragments more than 1000 bp, while FIELD BLOOD SAMPLING, section III:D is particularly efficient for short fragments up to 500 bp and when the filter paper cut is small (2 × 2 mm).

References

Evengård B, Linder E, Lundbergh P. 1988. Standardization of a filter-paper technique for blood sampling. Ann Trop Med Parasitol 82(3):295-303.

Gil JP, Nogueira F, Strömberg-Nörklit J, Lindberg J, Carrolo M, Casimiro C, Lopes D, Arez AP, Cravo PV, Rosario VE. 2003. Detection of atovaquone and Malarone resistance conferring mutations in *Plasmodium falciparum* cytochrome b gene (cytb). Mol Cell Probes 17(2-3):85-89.

Kain KC, Lanar DE. 1991. Determination of genetic variation within *Plasmodium falciparum* by using enzymatically amplified DNA from filter paper disks impregnated with whole blood. J Clin Microbiol 29(6):1171-1174.

Plowe CV, Djimde A, Bouare M, Doumbo O, Wellems TE. 1995. Pyrimethamine and proguanil resistance-conferring mutations in *Plasmodium falciparum* dihydrofolate reductase: polymerase chain reaction methods for surveillance in Africa. Am J Trop Med Hyg 52(6):565-568

Sakihama N, Mitamura T, Kaneko A, Horii T, Tanabe K. 2001. Long PCR amplification of *Plasmodium falciparum* DNA extracted from filter paper blots. Exp Parasitol 97(1):50-54.

Uchida JY, Kasahara T, Bobogare A, Saefafia S, Kere N, Kawabata M, Ohta N, Ishii A. 1995. The prevalence of *falciparum* malaria in the Solomon Islands investigated by a filter paper disk PCR method. Jpn J Parasitol 44(2):119-127.

Wooden J, Gould EE, Paull AT, Sibley CH. 1992. *Plasmodium falciparum*: a simple polymerase chain reaction method differentiating strains. Exp Parasitol 75(2):207-212.

IV. Postdose drug level monitoring

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Using the filter-paper samples, chromatographic analytical methods were developed to monitor capillary blood levels of the drug and its metabolites after administration by Bergqvist and colleagues. Currently methods for the following drugs are available:

References

Bergqvist Y, Churchill FC, Mount DL. 1988. Determination of mefloquine by electron-capture gas chromatography after phosgene derivatisation in biological samples and in capillary blood collected on filter paper. J Chromatogr 428(2):281-290.
Bergqvist Y, Funding L, Kaneko A, Krysèn B, Leek T. 1998. Improved method for the simultaneous determination of proguanil and its metabolites by high-performance liquid chromatography and solid-phase extraction of 100-μL capillary blood samples dried on sampling paper. J Chromatogr B 719(1-2):141-149.
Bergqvist Y, Hjelm E, Rombo L. 1987. Sulfadoxine assay using capillary blood samples dried on filter paper-suitable for monitoring of blood concentrations in the field. Ther Drug Monit 9(2):203-207.
Lindegardh N, Forslund M, Green MD, Kaneko A, Bergqvist Y. 2002. Automated solid-phase extraction for determination of amodiaquine, chloroquine, and metabolites in capillary blood on sampling paper by liquid chromatography. Chromatographia 55(1-2): 5-12.
Lindström B, Ericsson O, Alvan G, Rombo L, Ekman L, Rais M, Sjö qvist F. 1985. Determination of chloroquine and its desethyl metabolite in whole blood: an application for samples collected in capillary tubes and dried on filter paper. Ther Drug Monit 7(2): 207-210.

DATABASES

I. PlasmoDB: a functional genomic resource for *Plasmodium* parasites

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OVERVIEW

PlasmoDB belongs to the Eukaryotic Pathogen Database Resources (EuPathDB), a National Institutes of Allergy and Infectious Diseases-funded Bioinformatics Resource Center. The primary mission of PlasmoDB is to integrate genomic sequence with genomic-scale functional data from a variety of sources and provide the global research community with intuitive tools to interrogate this data and facilitate hypothesis driven research. Designed to make bioinformatics easily accessible to the bench scientist, PlasmoDB offers over 70 ways to search the underlying data in a user-friendly, graphic web-interface and is updated bimonthly with new data or new searches. Each annotated genomic feature (e.g. gene, EST) is represented by an ID, which links to a record page that contains all of the database information for that genomic feature. Users can study and compare the record pages of individual genomic features or they can perform their own *in silico* experiments using the search strategy system to assemble lists of records that share common biological characteristics.

The following manual describes the anatomy of PlasmoDB and provides step-by-step instructions to aid you in fully taking advantage of PlasmoDB. You are invited to read through this text or to go directly to these hyperlinked sections:

ACCESS: http://PlasmoDB.org

COMPONENTS OF PlasmoDB

PlasmoDB can be divided into 4 basic components: the database, the web interface, the record page, and the search strategy system.

The Database: Plasmodium genome sequence, annotation and experimental data in one location.

Genomic sequence and annotation. PlasmoDB obtains sequence and annotation data directly from sequencing centers (GeneDB, JCVI), individual labs or sequence repositories (EMBL and GenBank). Sequence data is available as fully annotated or as assembled sequence data. Updated annotation of several *Plasmodium* genomes has been ongoing at GeneDB with direct input from the community via user comments on PlasmoDB gene pages. Table 1 outlines the genomic sequence content of PlasmoDB including information about the annotation status, sequence source and gene count. Since PlasmoDB is regularly updated, a current list of data content may be accessed online. For a complete list of data sources and citations for data contained in PlasmoDB please visit the data source section.

Table 1: Species represented and sequence integrity in PlasmoDB

Species	Strain	Annotation available	Data Source	Available Megabase pairs	Gene Count
P. berghei	ANKA	Yes	Sanger	18.52	4970
P. chabaudi	chabaudi	Yes	Sanger	18.83	5182
P. cynomolgi	B	Yes	Genbank	26.18	5776
P. falciparum	3D7	Yes	Sanger	23.26	6372
	IT	Yes	Sanger	23.26	5519
	Dd2				N/A
	HB3				N/A
	C10 plastid only	Yes			
	NF54 mitochondrial only	Yes			
P. gallinaceum			Sanger	16.93	N/A
P. knowlesi	H	Yes	EMBL	23.73	5244
P. reichenowi			Sanger	7.38	N/A
P. vivax	Sal_1	Yes	Genbank	27.01	5507
	Sal_1 mitochondrial only	Yes			3
P. yoelii	17XNL	Yes	Genbank	22.94	7774
	YM	Yes	Sanger	22.03	5811

Data. The database contains genomic-scale data concerning gene expression, protein expression and biomolecular interactions. The functional genomics data in PlasmoDB is obtained from researchers worldwide and integrated into the database pre- or post- publication after evaluation and approval of the data provider. Since these datasets correlate with gene sequences, PlasmoDB displays functional genomics data in context with the genome and, in some cases, offers ways to search the datasets. The following descriptions include information about how the data is presented on PlasmoDB as well as examples of searches specific to that data type. Table 2 shows the type of data available for each plasmodium species. Data in PlasmoDB includes:

Microarray: glass slides or high-density array data.
PlasmoDB reanalyzes microarray data using a standard analysis method to ensure consistent representation of all microarray data on our site. Microarray data for individual genes are displayed as graphs and tables in the expression section of record pages. In addition, mapped probes for each microarray platform can be viewed in the genome browser. Depending on the original microarray experiment, PlasmoDB offers searches based on fold induction, percentile expression or similarity of expression. These searches return genes whose expression pattern satisfies the chosen search parameters.

Proteomics: peptides identified based on mass spectral analysis.
Peptide sequences are mapped to translated genes and tabulated or displayed graphically on gene record pages in the protein section. Mapped peptides from proteomics experiments are also available as data tracks in the genome browser. Searches based on proteomics data (peptide presence or spectral count) return genes whose expression was confirmed by mass spec peptides.

ChIP-chip: microarray data using chromatin immunoprecipitated DNA.
ChIP-chip data are presented as expression values associated with sequences that represent DNA-protein interactions. ChIP-chip data can be displayed in the genome browser. Searching for genes based on ChIP-chip returns genes based on their proximity to ChIP-chip peaks.

Chip-Seq: high throughput sequencing of chromatin immunoprecipitated DNA
Sequences representing DNA that was bound to protein are mapped to the genome and displayed as tracks in the genome browser.

RNA-Seq: High throughput sequencing data.
Sequence reads are mapped to the genome. Expression graphs and tables are available on gene pages and tracks representing depth of coverage can be viewed in the genome browser. In addition, splice junction sites based on intron spanning reads that provide evidence for gene structure can be displayed as a track in GBrowse. Searching for genes based on RNA-Seq depth of coverage returns differentially expressed genes.

TF binding site evidence: protein binding microarray data.
Sequences representing transcription factor (TF) binding sites can be displayed in the genome browser. Searching for genes based on TF binding site evidence returns genes based on the presence of an AP2 transcription factor-binding site in a defined location relative to genes.

ESTs: (Expressed sequence tags): sequence data from cloned cDNA libraries:
EST data is retrieved from the GenBank Expressed Sequence Tag database (dbEST) and mapped to the genome. EST alignments are available as a data track in the genome browser. Searching for genes based on specific EST libraries returns genes that have ESTs mapped to them. In addition, several searches return ESTs based on their GenBank accession numbers, BLAST, genomic location, list of gene IDs, extent of gene overlap and library (or sample).

SAGE Tags (Serial Analysis of Gene Expression): sequences representing expressed transcripts.
SAGE data is mapped to the genome and tabulated in the expression section of gene record pages or displayed as a track in GBrowse. Searching for genes based on SAGE data returns genes that are expressed in the samples tested or that are differentially expressed between libraries. In addition, several searches return SAGE tags records based on SAGE Tag IDs, Gene IDs, genomic location, tag sequence, expression level and differential expression.

SNPs (Single Nucleotide Polymorphisms): SNP calls based on microarray or sequencing data. SNPs are tabulated and displayed graphically on gene pages or in the genome browser. Search for genes based on their SNP characteristics (ie.

synonymous, nonsynonymous or noncoding). In addition, several searches return SNPs based on their IDs, gene IDs, allele frequency, genomic location, isolate comparison and presence in an isolate assay.

Isolates: genotype and meta data such as geographic location.
Plasmodium samples collected in the field are genotyped using a variety of methods including sequencing, microarray, or barcode PCR. Isolate sequences aligned to the genome can be visualized in GBrowse. Isolate records include all available meta data. Several searches return isolates based on a variety of criteria including isolate ID, taxon/strain, host, isolation source, locus sequence, geographic location, study authors and BLAST similarity, text and isolate clustering.

Pathways: hierarchical listing of terms describing the biological process, and molecular function of the gene product.
Gene Ontology (GO), enzyme commission (EC) and metabolic pathway information is gathered from several sources and incorporated into record pages as appropriate. In addition, searches are available to allow the identification of genes based on GO terms and EC numbers or to identify lists of genes that belong to a specified metabolic pathway.

Protein Interactions: Yeast two-hybrid and predicted functional interaction data.
Interaction data is also displayed on gene pages. Searching for genes based on Y2H protein interactions returns genes that are interacting partners with your specific gene of interest.

Subcellular localization: Exported proteins and apicoplast targeted proteins.
Proteins that are predicted to be exported into the red blood cell or targeted to the apicoplast may be identified.

Data Generated from In-house Analyses: Several types of data are generated using standard analysis of sequence data. Detailed analysis methods are available in the analysis methods section of PlasmoDB. In addition, all analysis results are displayed on individual gene pages:

Predicted Signal Peptide: predictions are made with the SignalP program.
Searching for genes based on the presence of predicted signal peptide returns genes whose sequences are predicted to encode a signal peptide.

Transmembrane Count: transmembrane domains are predicted using TMHMM2.
Searching for genes based on transmembrane count returns genes with a user-specified number of predicted transmembrane domains.

Molecular Weight: computationally calculated from the raw translation of the gene sequence. Searching for genes based on molecular weight returns genes whose translated protein products have calculated molecular weights within a user-specified range.

Isoelectric Point: computationally calculated from the raw translation of the gene sequence using the EMBOSS package.
Searching for genes based on isolelectric point returns genes whose translated protein products have a calculated isolelectric point within a user specified range.

Protein Secondary Structure: secondary-structure contents are calculated with PSIPRED. Searching for genes based on the presence of predicted secondary structure returns genes whose proteins are predicted to have a certain (user-defined) amount of helix, coil, or strand.

Protein Database 3D Structures: Protein sequences are compared to sequences with structure in the Protein Data Bank.
Searching for genes based on their PDB 3-D Structures returns genes whose protein products show similarity to known structures in the Protein Data Bank.

Predicted 3D Structure: Computationally generated 3D structure models seeded with structures from PDB.
Searching for genes based on their predicted 3D structure returns genes that have computationally predicted 3D structure models.

Open reading frames (ORFs): Predicted ORFs 50 amino acids or greater based on a six frame translation of genomic and EST sequences.
ORFs can be identified based on their IDs, presence of a specific amino acid motif, BLAST similarity or genomic location. ORFs can also be visualized on genomic and EST sequence pages or in GBrowse.

Synteny: Syntenic genes and regions between *Plasmodium* species are computed based on exon coordinates and orthology. Synteny is displayed on all gene pages and is available as a data track in GBrowse.

Orthology Group: Genes are grouped into orthology groups based on the OrthoMCL database. The results of any search conducted on a specific *Plasmodium* species can be transformed to their orthologs in all other species in PlasmoDB. Searching for genes based on their phylogenetic pattern returns genes based on their inclusion in ortholog groups (e.g. find all conserved *Plasmodium* genes that do not have orthologs in mammals).

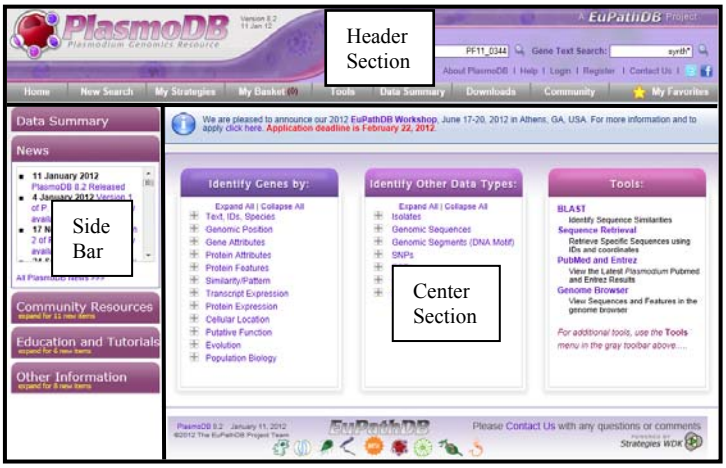
Table 2. Functional genomics data available for each species.

Species	Microarray	Proteomics	ChIP chip	Chip Seq	RNA Seq	Isolates	ESTs (libraries)	SAGE tags
P.berghei	2 expts	1 expt				121	17	
P.chabaudi						121		
P. cynomolgi						103		
P.falciparum	8 expts	10 expts	3 expts	2 expts	4 expts	21510	>100	3 expts
P.gallinaceum								
P.knowlesi						410		
P.reichenowi						74		
P.vivax	1 expt	2 expts				11732	4	
P.yoelii	1 expt	1 expt				151	8	

The Web Interface: accessing PlasmoDB.

The PlasmoDB web-interface is designed to provide easy, intuitive access to our searchable database. Mouse-over menus, mouse-over popup windows and hyperlinked text are incorporated into the site to lead users to accurate information. The following sections

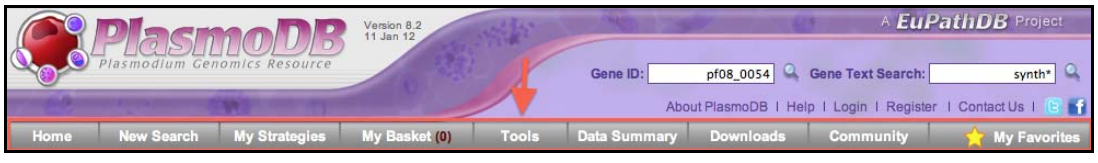
The Home Page: www.plasmodb.org.



The header section, available on any PlasmoDB webpage, contains quick search boxes links, and a gray menu bar. The center section contains links to searches and tools. The side bar has links news, tutorials and other resources. Highlights from each section are described below. Please

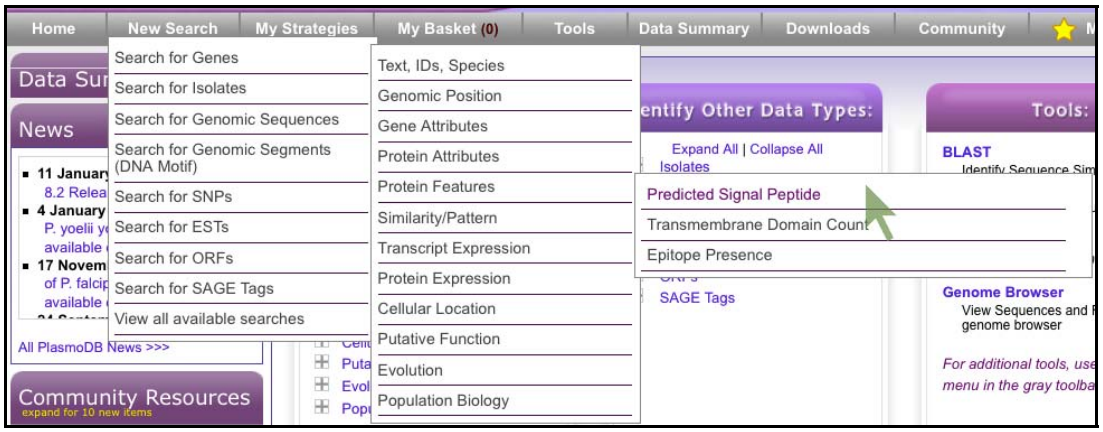
The Header Section:

- The **PlasmoDB and EuPathDB logos** serve as links to their respective home pages. The two digit version number and date appear to the right of the PlasmoDB logo. Versions numbers advance to the next whole number (7.2 becomes 8.0) when a release incorporates new or re-annotated genomes. The decimal advances when a release incorporates new data, new searches or new analysis features.
- The **Gene ID and Gene Text Search** boxes offer the quickest way to access these searches. Enter a Gene ID in the Gene ID search box, click the search icon and you will automatically be taken to the gene record page. Enter text in the Gene Text Search box to quickly search for genes using PlasmoDB's predetermined text search parameters. Genes whose records contain the text phrase will be returned.
- Under the quick search boxes are **links to register and login to PlasmoDB**. You do not have to register to use the site but registered users can save or share search strategies, make comments on record pages, and use the basket and My Favorites features. Unregistered users lose their searches upon closing the browser.
- Clicking the **Contact Us** link opens a form for submitting questions, error reports, feature requests and dataset proposals. **We make every effort to answer requests quickly**.
- The **gray menu bar** is a mouse-over menu that can be used to reach all searches (New Search), your current and saved searches (My Strategies), as well as your My Basket and My Favorites pages. Useful features of the gray menu bar are highlighted below (arrow).

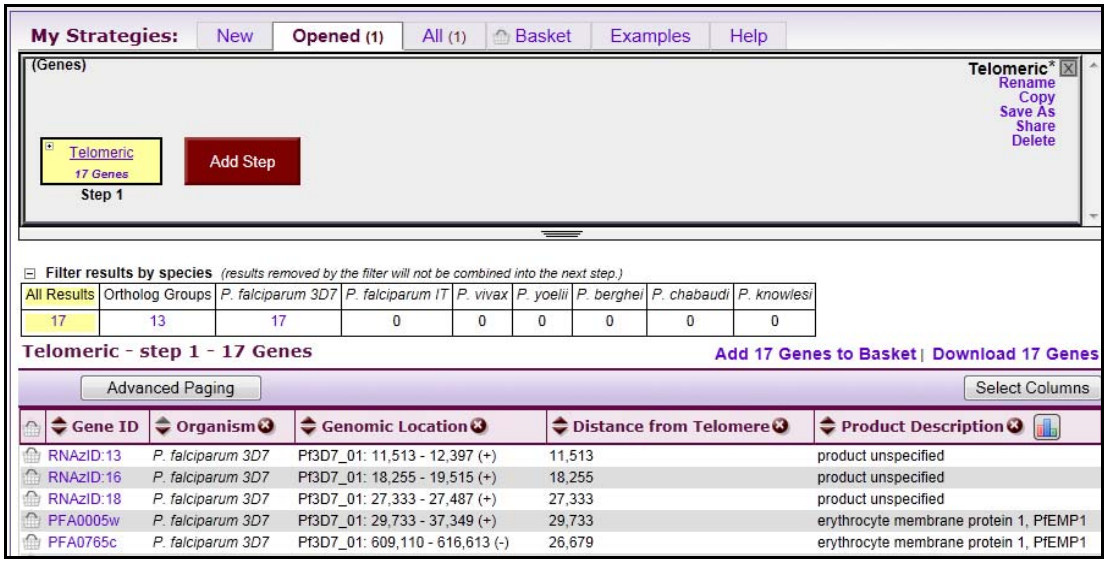


Home: This link returns to the home page.

New Search: Mouse over the "New Search" tab to bring up an expandable menu of searches. Click the last option in the first menu "View all available searches" to open a page that has links to every PlamoDB search.



My Strategies: Click this tab to open your "My Strategies" page where search results are displayed. When you run a search, the "My Strategies" page automatically opens to show the results. The "My Strategies" page contains six tabbed subpages:



- New: Click this to open the "All Available Searches" page and begin a new search.
- Opened: Displays the strategies you have run during your current session.
- All: The "All" tab in your My Strategies page keeps track of all the searches you have run, even searches from previous sessions if you are a registered user. Unregistered users lose their searches upon closing the browser.
- Basket: The Basket allows registered users to assemble lists of records by clicking on the basket icon in the gene page or result table. Handpicking records in this way allows the user to bypass the search strategy system when refining a result list.
- Examples: This page contains links to example strategies.
- Help: This page defines some useful functions within the search strategy system and contains a link to a tutorial about building strategies.

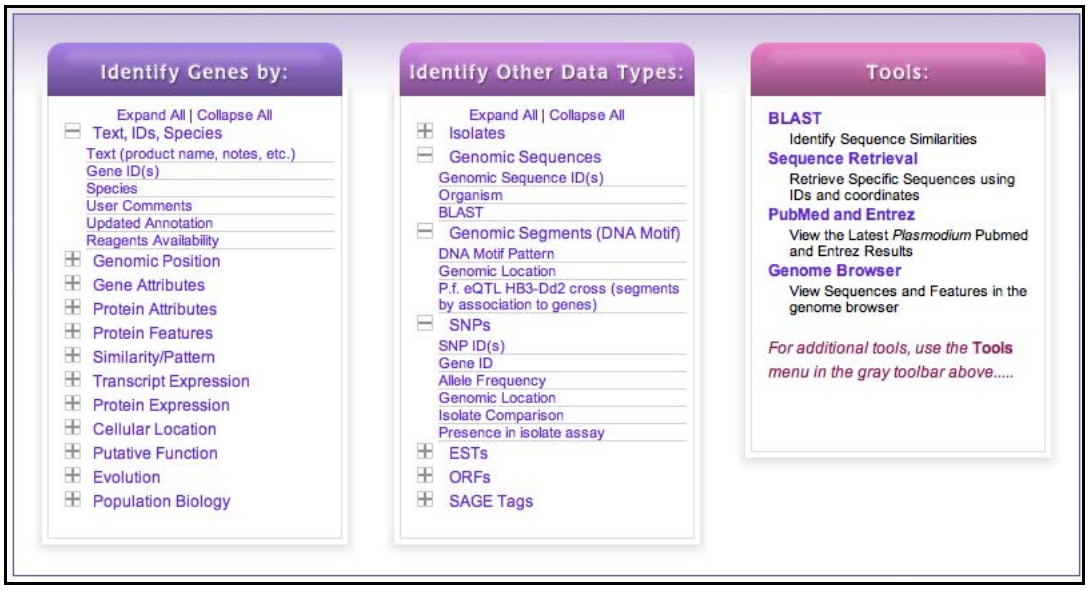
My Basket: Registered users have the option to assemble a list of handpicked individual records. The basket is intended as temporary storage for short lists of records that will be sent to the search strategy system.

Data Summary: "Data Summary" links to a page describing the source of datasets in the database, a description of analysis methods used to generate data for our searches, a table describing the genomes and data types available, and a Gene Metrics table that summarizes the number of gene and other data available.

Downloads: Sequence and data are available for download in FASTA, .gff, or text file format. Files added by users and containing useful and relevant information concerning the Plasmodium field are also offered for download.

My Favorites: Registered users can bookmark record pages so they can be quickly accessed. The "My Favorites" page is a permanent personal storage area for registered users.

The Center Section: The Center section contains links to PlasmoDB's prebuilt searches and to useful tools. Searches are organized by the genomic feature (record type) returned by the search.



- The **"Identify Genes by"** section is an expandable table of **links to all PlasmoDB searches that return gene record pages.** Click on the plus sign or section title to expand the section. Go to a search form by clicking any option within a section.
- The **"Identify Other Data Types"** section is an expandable table of **links to all PlasmoDB searches that return record types that are not genes**.
- The **"Tools"** section contains links to our BLAST search page, our Sequence Retrieval Tool, the results page for PubMed or Genbank searches for "plasmodium", and a link to our GBrowse application.

The Side Bar: The side bar links to our Data Summary table, our new releases, community resources, an education and tutorials section and other information.

- The **data summary table** describes the types of data available for every species in PlasmoDB and other EuPathDB sites.
- Our **news releases** are generated with each bimonthly database release. The documents describe new data, new searches and new analysis tools that appear on the site database release.
- Under **"Education and Tutorials"** are links to our web tutorials, our workshop home page, exercises from the workshop, NCBI's Glossary of terms, our glossary, and a link for contacting us.

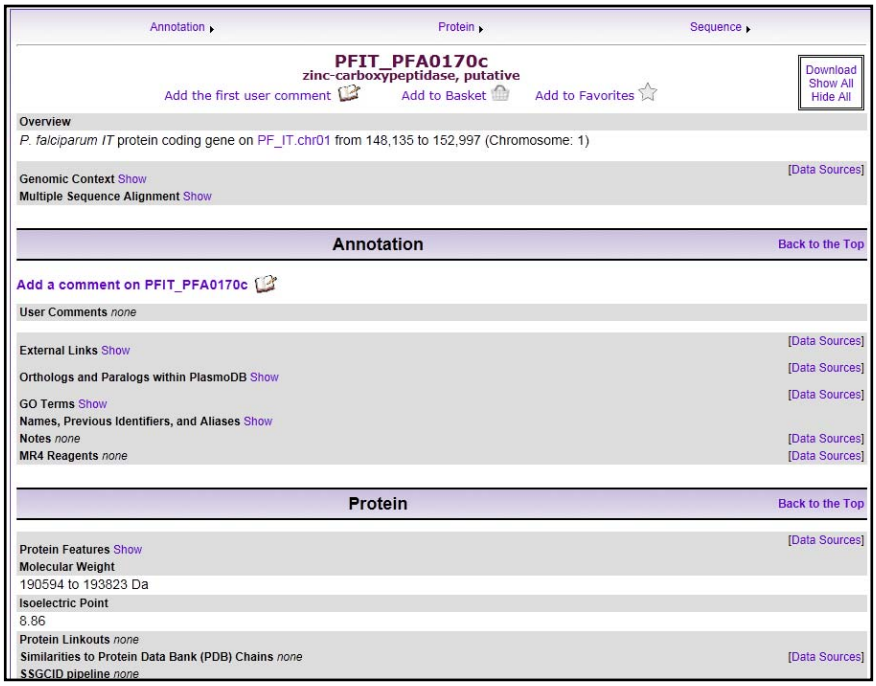
The Record Page: PlasmoDB's basic unit of information for a genomic feature.

A record page is the complete subset of database information that refers to one specific genomic object. Each record page is a list of links to data. Data is presented in tabular or graphic form. PlasmoDB defines seven types of record pages: genes, SNPs, ESTs, ORFs, SAGE tags, genomic sequences, and isolates. Each record type represents a class of genomic object or feature for which PlasmoDB builds searches. At the top of every record page is the record ID. The box in the upper right corner of a record page offers options to "Download" the data, and open or close all the data links on the record page with the "Show All" and "Hide All" options.

Please follow the hyperlinked titles to open an example of the respective record page.

1. Genes:

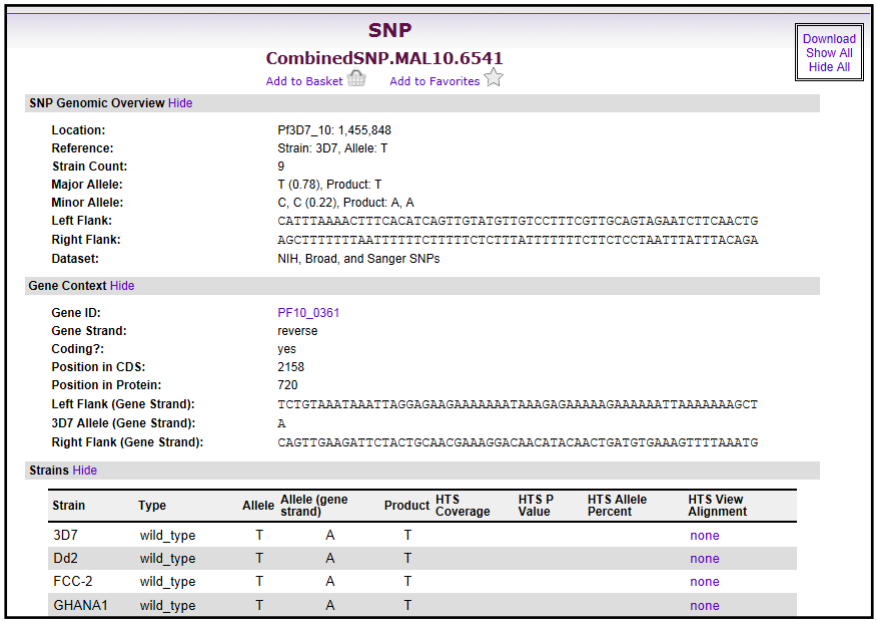
The record page of a gene contains 5 sections: overview, annotation, protein, expression and sequence. The "Genomic Context" graphic shows the gene in context with the genome and includes synteny and SNP information available for the gene. The same type of graphic is available for the protein product under "Protein Features".



The Annotation section of gene record pages contains a link to **Add a comment on the gene.** These comments appear immediately on PlasmoDB and searchable using the gene text search. User comments are monitored by the annotation centers so your comments serve a vital function in updating genome annotation. Comments can include any information you have learned about the gene during your work or information from reading publications.

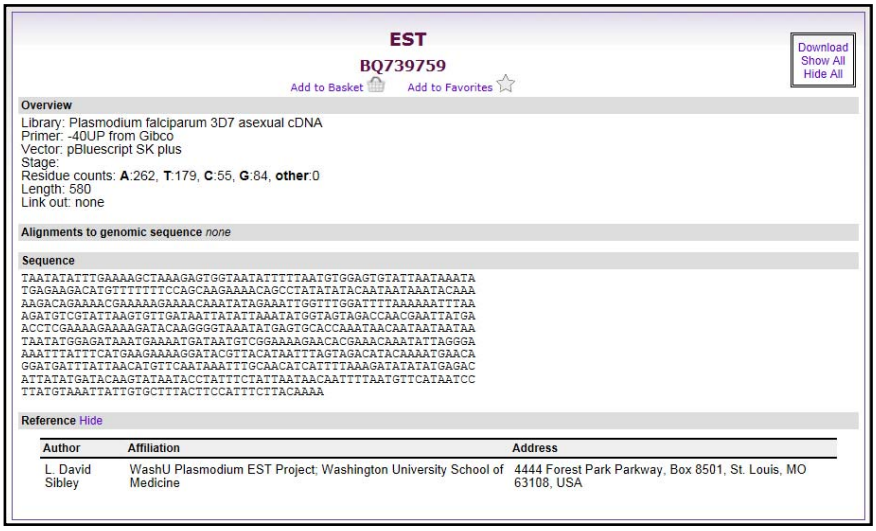
2. SNPs:

The SNP record page contains a SNP Genomic Overview, a gene context section, and several useful tables concerning strains, isolates, and other SNPs at the location.



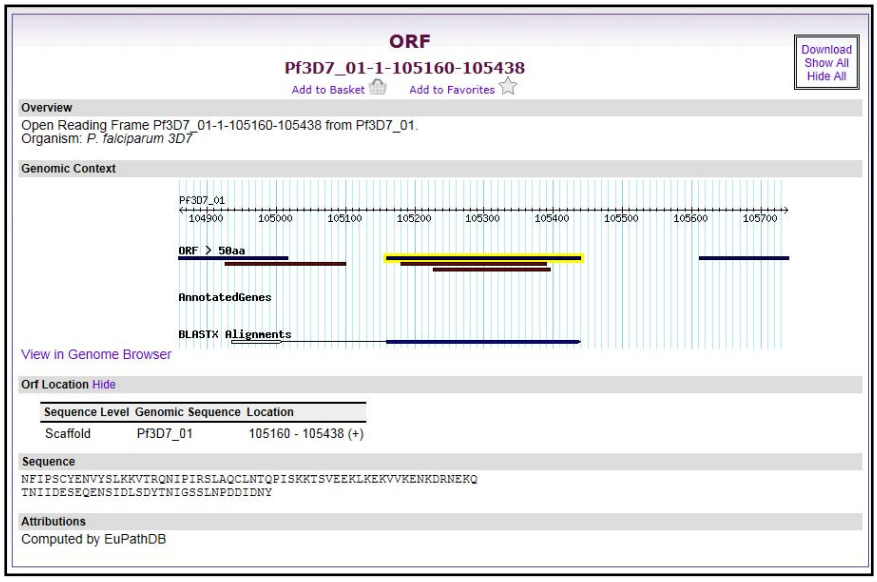
3. ESTs:

The EST record page includes an Overview and reference, and shows the sequence and alignments to genomic sequence.



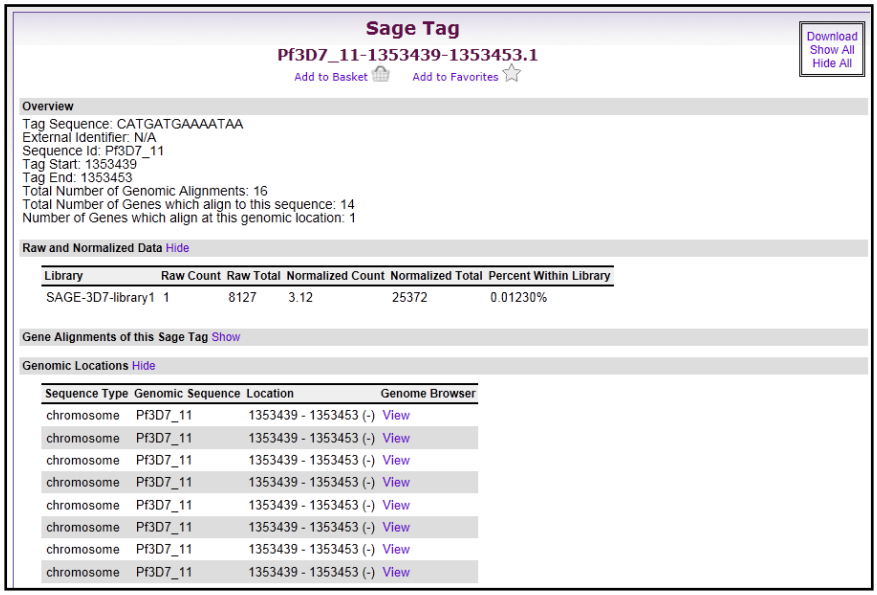
4. ORFs:

The ORF record page contains an overview, a Genomic context graphic, the location and the sequence.

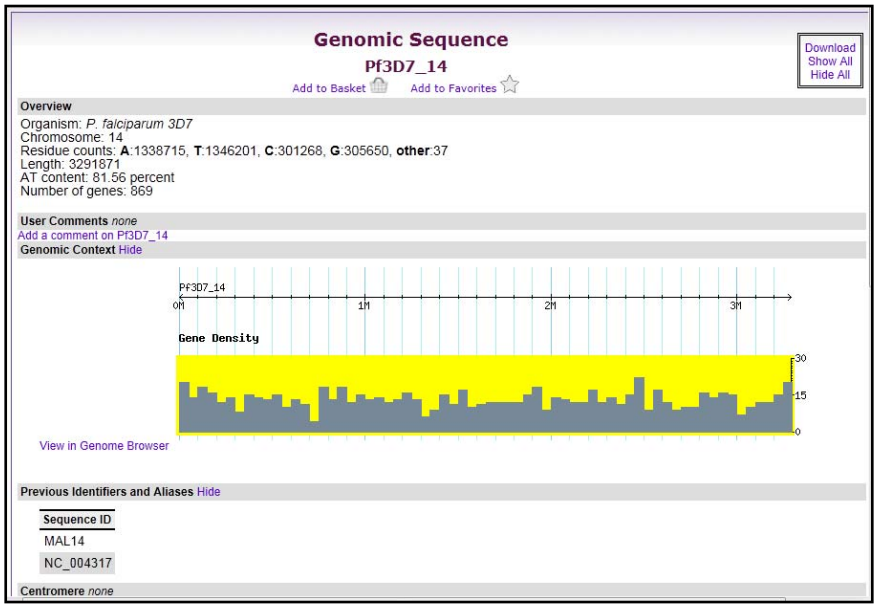


5. SAGE Tags:

SAGE Tag record pages contain and overview and several useful tables including “Gene Alignment of the Sage Tag” and “Genomic Locations”.



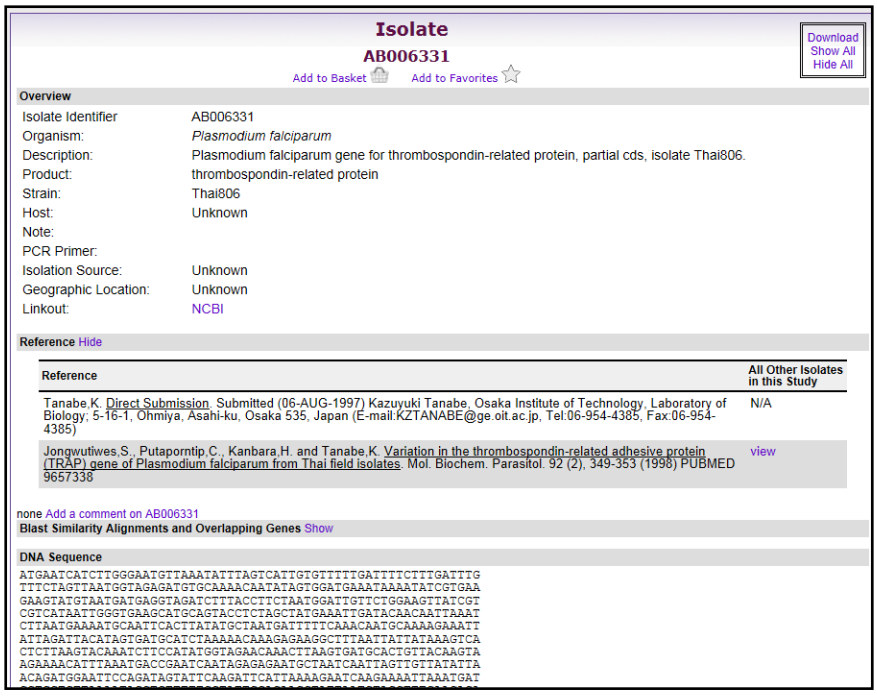
6. Genomic Sequences:



PlasmoDB defines "genomic sequence" as the largest assembled segments of sequence available for that organism. Sometimes our genomic sequences represent chromosomes, as in *P. falciparum*. Other times genomic sequences represent contigs, as in *P. gallinaceum*. The Genomic Sequence record page includes an overview, a genomic context graphic and several useful tables. Links for adding user comments concerning the genomic segment and for downloading sequence are also provided on the Genomic Sequence record page.

7. Isolates:

Isolate records contain an overview section, a reference, the sequence resulting from the genotyping, and a table of "blast similarity alignments and overlapping genes.



The Search Strategy System: pulling information from the database.

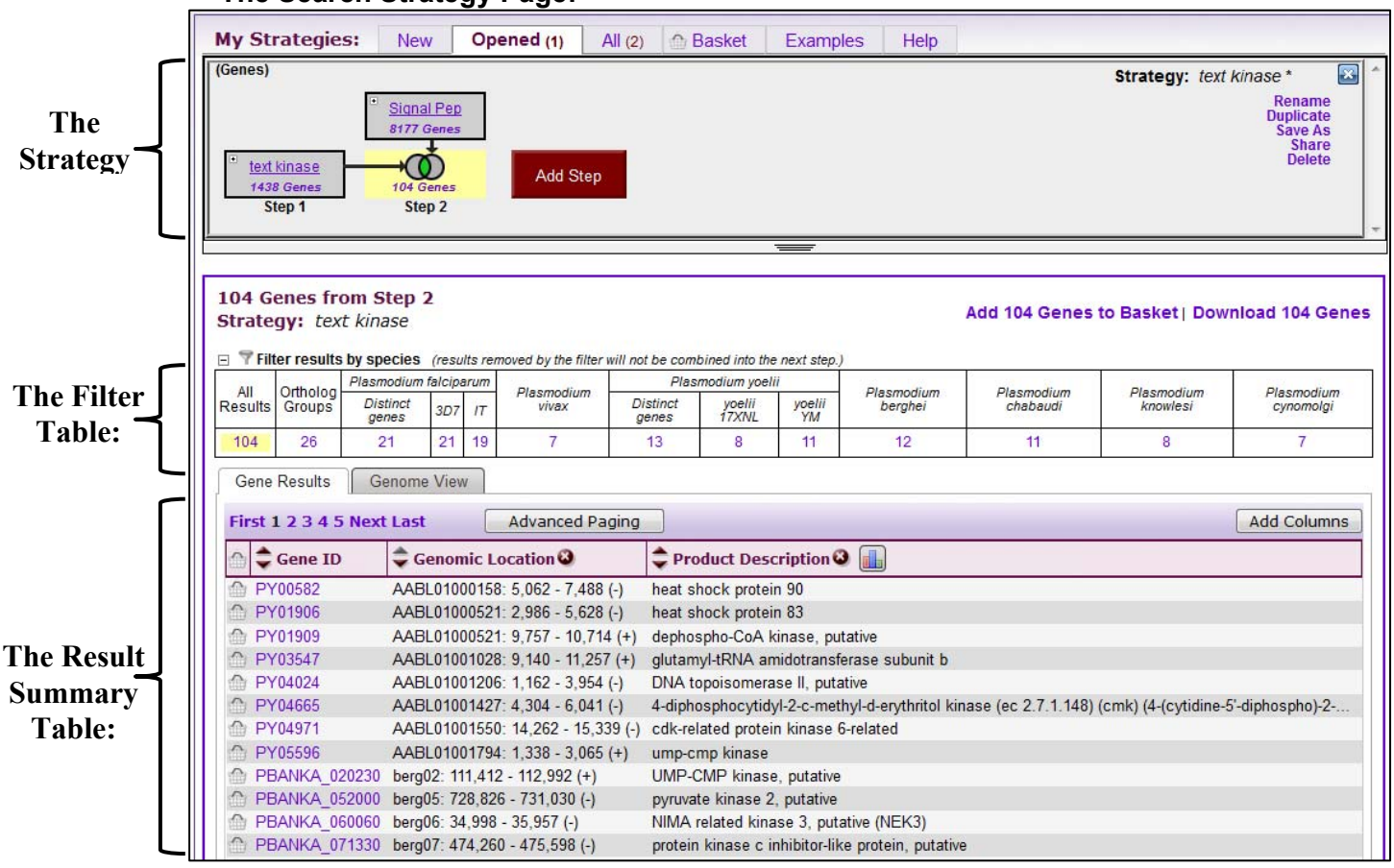
PlasmoDB offers more than 70 searches allowing users to identify genomic features that are annotated in the datasets. Each search is designed to look for records that share specific biological or experimental characteristics. Searches return a list of IDs for genomic features that match the user-defined characteristics. Each ID links to a record page that contains all the data in the database about that specific genomic feature.

A search strategy is a series of searches that are logically combined to refine the biological meaning of the result list. For example, a list of potentially secreted kinases can be assembled by a search strategy that intersects the result list of a text search for kinase, with a result list for genes that have predicted signal peptides.

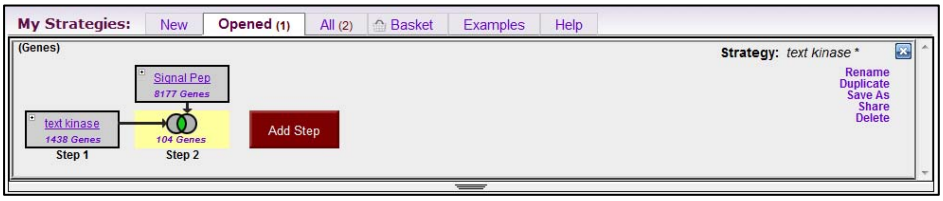
We organize our searches based on the record type that is returned by the search: genes, SNPs, ESTs, ORFs, SAGE tags, genomic sequences, or isolates, (see previous section describing record types). Searches are initiated from a search form that prompts the user to define or choose parameters that fix the boundaries of the biological characteristics of records returned by the search. Searches returning the same record type can be combined using minus, intersect, union or colocation. Searches returning different record types, genes and SNPs for example, can only be combined using colocate.

The Search Strategy page has three sections: the strategy builder used to combine searches, the filter table used to quickly toggle the result table between species, and the results table which lists IDs and other information returned from the search. The search strategy page is automatically opened after you run your first search.

The Search Strategy Page:

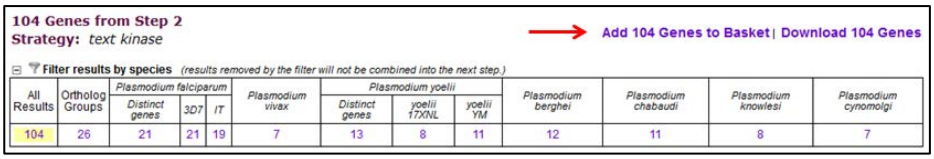


The Strategy Panel:



The strategy panel is a graphic interface that allows you to easily choose and combine successive searches. The graphic view of the strategy panel appears at the top of the result page of your first search. The search you just ran is presented as STEP 1 of a strategy. Each step box contains the search name/title and the number of hits returned. The box highlighted in yellow is the search whose results are used to populate the filter and results table. Click any step name (text kinase) to open that search and revise it. Click on any result summary (1438 Genes, or 104 Genes) to populate the filter and result tables with those results. You have the option to ignore the strategy panel and scroll down to the result table and look at your results.

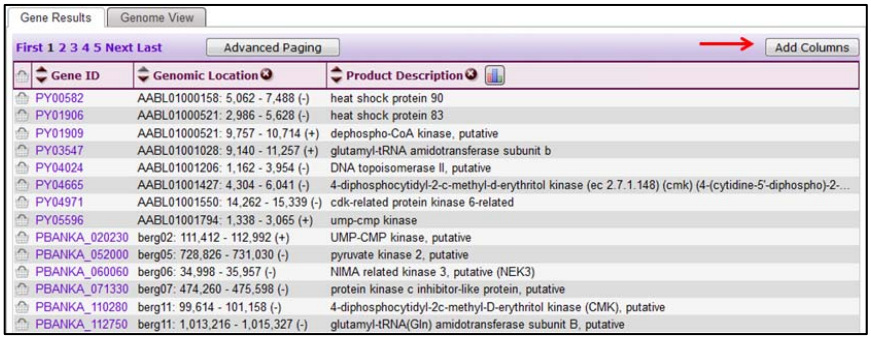
The Filter Table:



All Results	Ortholog Groups	Plasmodium falciparum			Plasmodium vivax	Plasmodium yoelii			Plasmodium berghei	Plasmodium chabaudi	Plasmodium knowlesi	Plasmodium cynomolgi
		Distinct genes	3D7	IT		Distinct genes	yoelii 17XNL	yoelii YM				
104	26	21	21	19	7	13	8	11	12	11	8	7

The filter table shows the distribution of hits across species and can be used to control the contents of the result table. Clicking on a hit number will limit the contents of the result table to IDs in that block. Above the filter table are links to add your results to your Basket or to Download them.

The Result Summary Table:



Gene ID	Genomic Location	Product Description
PY00582	AABL01000158: 5,062 - 7,488 (-)	heat shock protein 90
PY01906	AABL01000521: 2,986 - 5,628 (-)	heat shock protein 83
PY01909	AABL01000521: 9,757 - 10,714 (+)	dephospho-CoA kinase, putative
PY03547	AABL01001028: 9,140 - 11,257 (+)	glutamyl-tRNA amidotransferase subunit b
PY04024	AABL01001206: 1,162 - 3,954 (-)	DNA topoisomerase II, putative
PY04665	AABL01001427: 4,304 - 6,041 (-)	4-diphosphocytidyl-2-c-methyl-d-erythritol kinase (ec 2.7.1.148) (cmk) (4-(cytidine-5'-diphospho)-2-...
PY04971	AABL01001550: 14,262 - 15,339 (-)	cdk-related protein kinase 6-related
PY05596	AABL01001794: 1,338 - 3,065 (+)	ump-cmp kinase
PBANKA_020230	berg02: 111,412 - 112,992 (+)	UMP-CMP kinase, putative
PBANKA_052000	berg05: 728,826 - 731,030 (-)	pyruvate kinase 2, putative
PBANKA_060060	berg06: 34,998 - 35,957 (-)	NIMA related kinase 3, putative (NEK3)
PBANKA_071330	berg07: 474,260 - 475,598 (-)	protein kinase c inhibitor-like protein, putative
PBANKA_110280	berg11: 99,614 - 101,158 (-)	4-diphosphocytidyl-2c-methyl-D-erythritol kinase (CMK), putative
PBANKA_112750	berg11: 1,013,216 - 1,015,327 (-)	glutamyl-tRNA(Gln) amidotransferase subunit B, putative

The example to the left shows 3 columns:

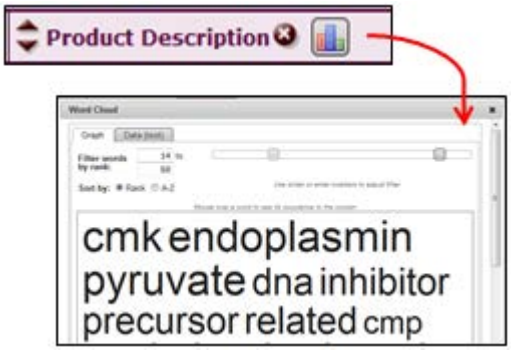
Basket icon: used to send this gene to your basket.

Gene ID: The Gene ID is a unique identifier for that gene record. Clicking the gene ID will open the record page for that gene.

Genomic Location: notations for the chromosomal location of the gene.

Product description: If information about the gene product is known, it will appear in this column.

Add Columns: The result table is presented at first with the fewest necessary columns of information. Add columns to the table by clicking “Select Columns” and following the popups.



The Column Analysis Tool:
Clicking on the bar chart icon next to a column name will open a window that allows for the analysis of data in that column. Columns of text can be analyzed by word cloud and other features will be available in the future.

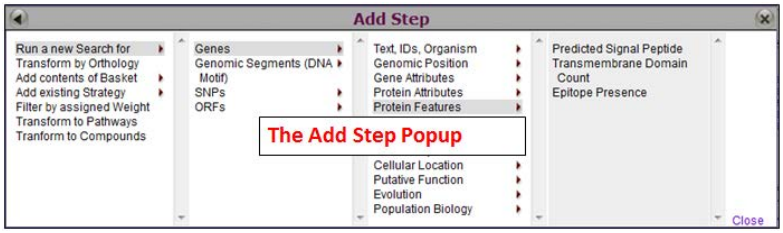
HOW TO RUN A SEARCH:

Each search is initiated from a search form page that can be navigated to from the center section of the home page, the drop down menu of the header section or from the all searches page. The search page will prompt you to define parameter values or enter information that will be used during the search. Clicking "Get Answer" from the bottom of the search form initiates the search and the results are automatically displayed once the search has finished.

HOW TO BUILD A STRATEGY:

To begin, choose your first search from the center section of the home page, from the all searches page, or from the mouse over menu at the New Search tab. Subsequent searches are chosen by clicking "Add Step" and following the options in the Add Step popup window. The "Add Step" popup window offers choices to extend the strategy by: running a new search, transforming by orthology, adding the contents of a basket, adding an existing strategy, or filtering by an assigned weight. Following the menus offered in the Add Step popup leads to the full array of searches available from the home page and mouse over menus used to run your first search.

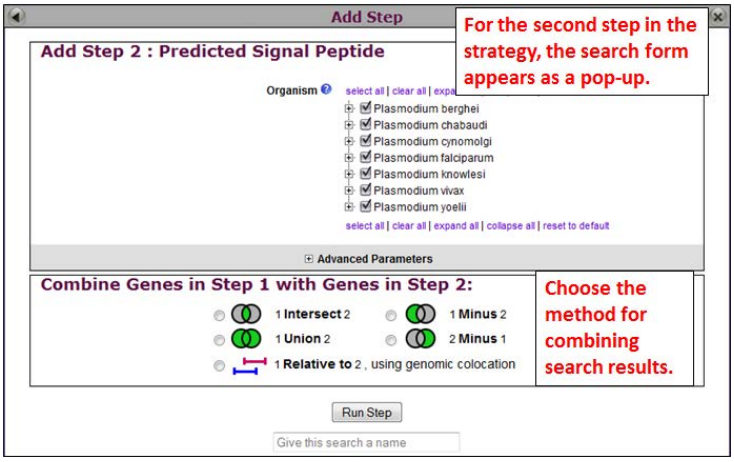
Once a new search is chosen, the search form appears as a popup window. The user is prompted to choose parameters for the new search as well as the method of combining the search results. Search results can be combined 5 different ways.



1 Intersect 2: The result lists of search 1 and 2 are compared and IDs that occur in both lists are returned as the intersected list.

Union: The result lists of search 1 and 2 are combined and returned as the result list. IDs that appear in both lists only appear once in the new result list.

1 Minus 2: The IDs that appear in both lists are removed from list one. The combined result list will contain only IDs that are unique to result list 1.



2 minus 1: IDs that appear in both lists are removed from list 2. The combined result list will contain only IDs that are unique to Step 2 results.

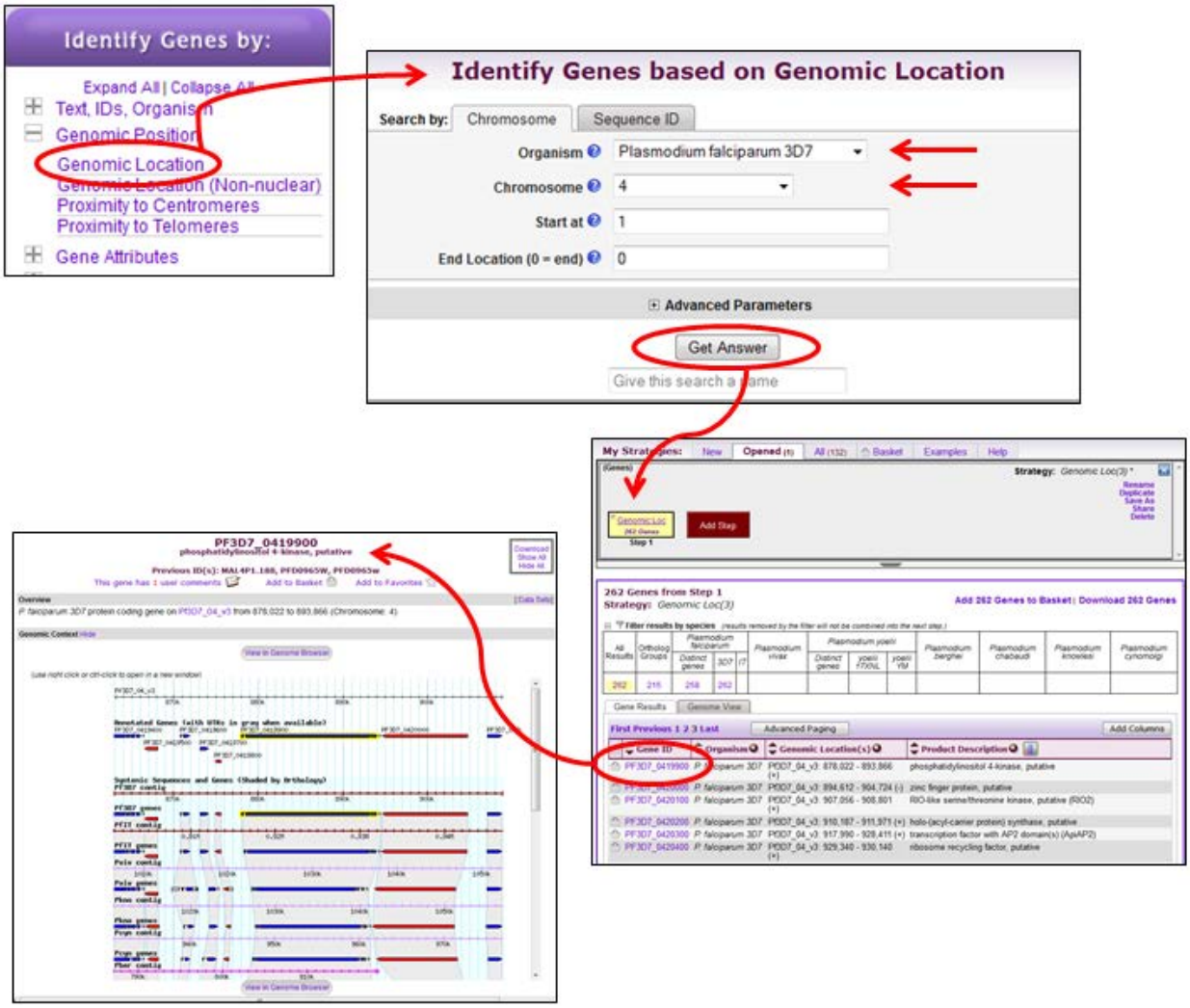
1 Relative to 2, genomic colocation: The genomic location of each ID in the result list of step 1 is compared to the genomic location of each ID in the result list of step 2. The combined result list will contain ID's that are within a user-defined distance from one another. This method involves an intermediate popup window for defining the colocation parameters, and this tutorial is helpful.

HANDS ON EXAMPLES

EXAMPLE 1: Find all genes on chromosome 4 of *P. falciparum* 3D7 and visit a gene page.

1.1Run the search “Identify Genes by Genomic Location”.

1.1.1. Navigate to the search form, choose your organism and chromosome and click “Get Answer”.
1.1.2. The result page automatically opens showing you the strategy window, the filter table and the result table. Click any ID in the result table to navigate to that record page.

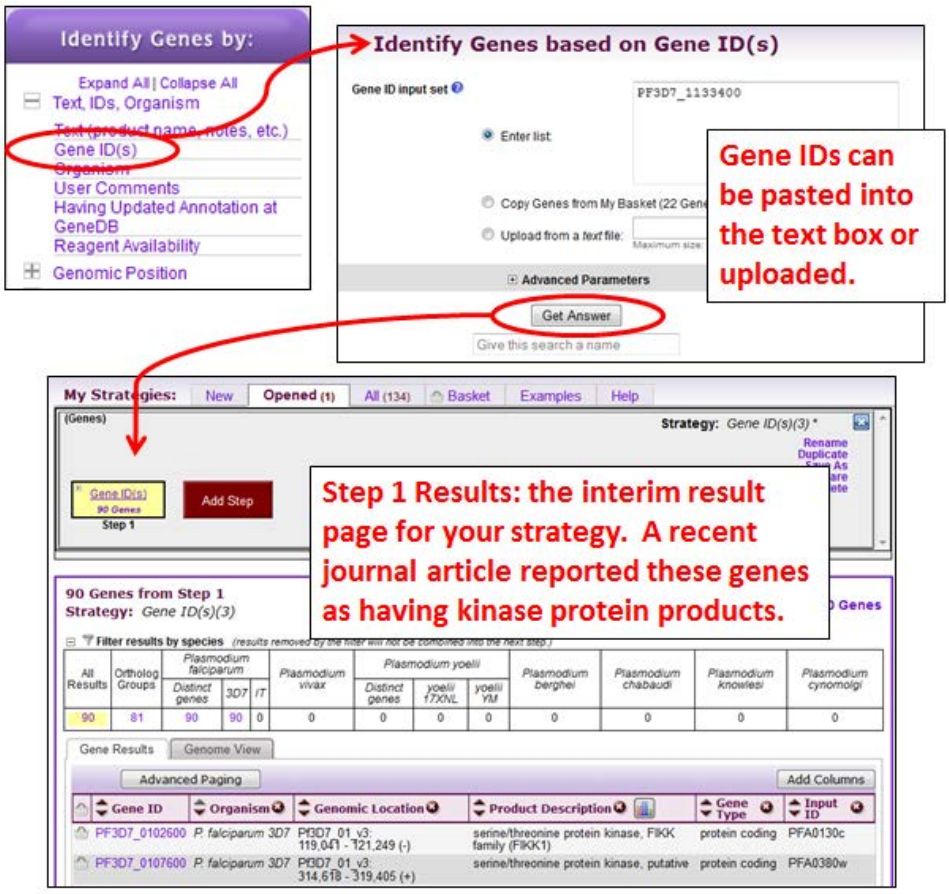


EXAMPLE 2:
You are interested in membrane bound kinases. A study analyzing the kinomes from organisms across the phylum Apicomplexa (*BMC Evolutionary Biology* 2011, 11:321) **containes a table of kinases from *P. falciparum*. Use PlasmoDB to determine what portion of the *P. falciparum* genes in the publication are possibly membrane bound.**

2.1 **STEP 1 OF YOUR STRATEGY**: Assemble a list of IDs that correspond to the genes mentioned in the literature reference using the "Identify Genes by Gene ID" search.

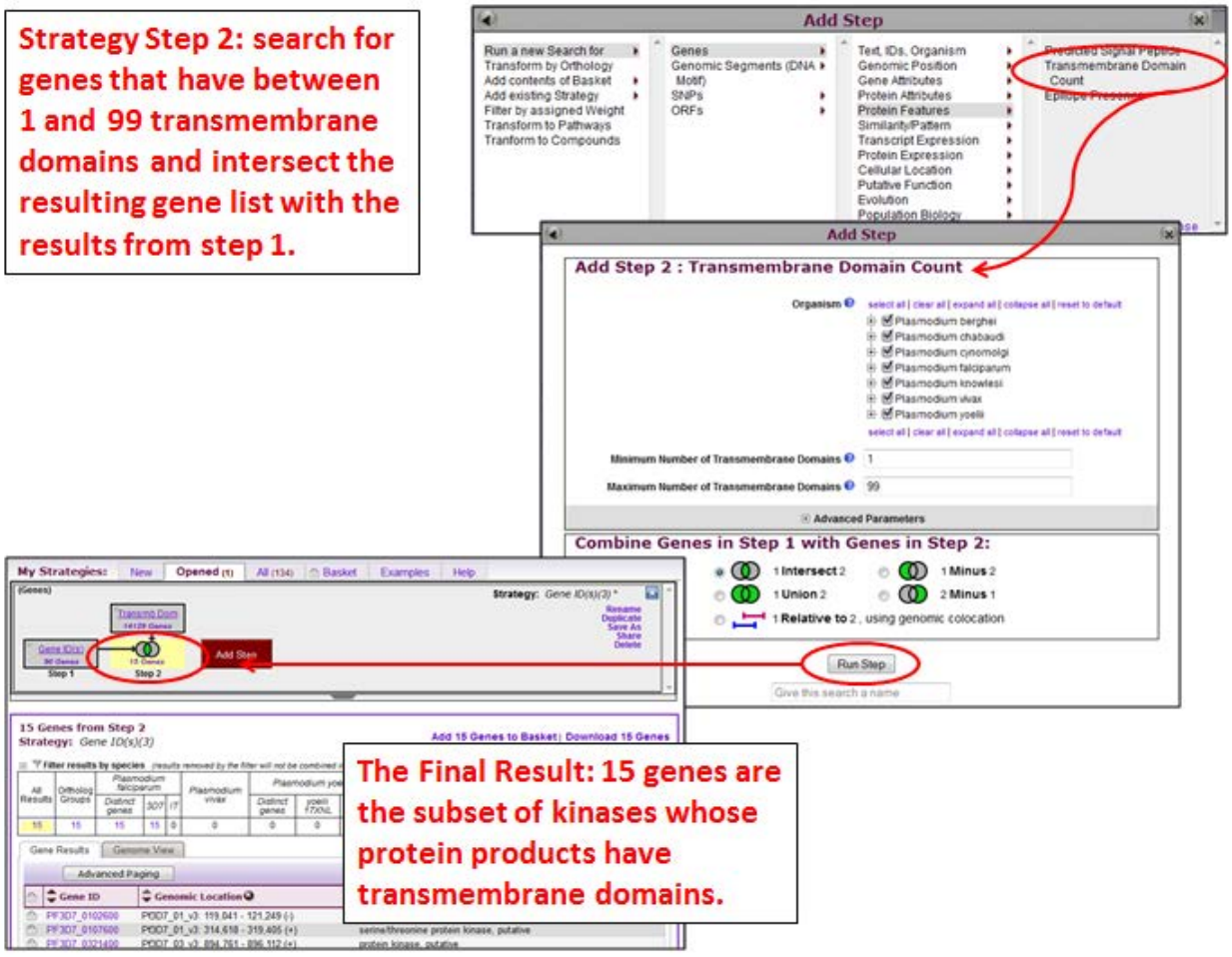
2.1.1.To retrieve the list of kinase gene IDs from the supplemental information from the paper, go to http://www.biomedcentral.com/1471-2148/11/321 and download/extract the "Additional Information 1" files. The file named "a1_Plasmodium-falciparum" is a spreadsheet that contains 93 gene IDs in column A.

2.1.2.Search the database for the genes represented by these IDs using the "Identify Gene by Gene ID". Cut and paste the gene IDs from the reference into the text box on the search page and click "Get Answer".
Note: The *P. falciparum* genome has been re-annotated since the paper was published. Gene IDs were changed to include a strain designation and some gene IDs were retired or combined if there was evidence that the gene model was wrong. The IDs you downloaded from the "a1_Plasmodium-falciparum" are from the old naming system but our system will map old IDs to new ones. The 93 gene IDs in the old annotation now map to 90 gene IDs.



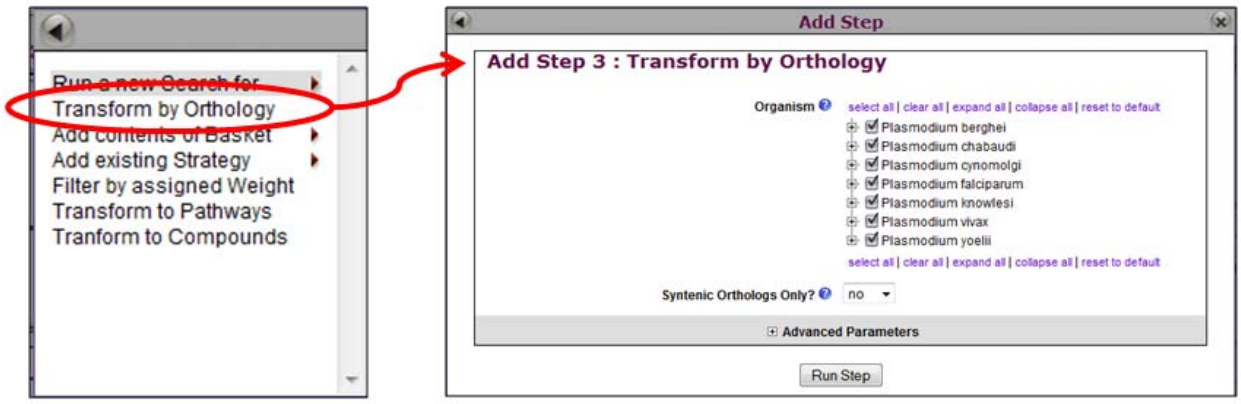
2.2 STEP 2 OF YOUR STRATEGY: Determine which of these 90 genes have a transmembrane domain.

2.2.1 Click “Add Step” and follow the choices in the popup window to the search “Identify Genes by Protein Features and Transmembrane Count”.

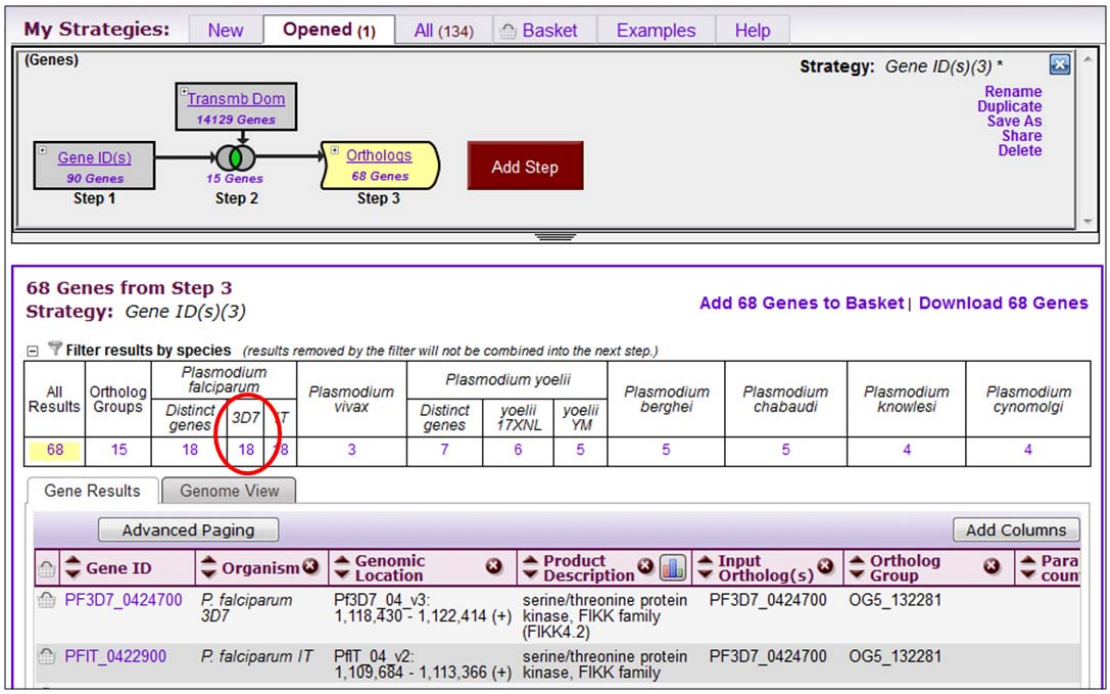


EXAMPLE 3: Leverage orthology to expand your results to all *Plasmodium* species in PlasmoDB. In this exercise we will take the results from the example 2 above and transform them to all *Plasmodium* species in PlasmoDB.

3.1 Start with the search strategy from example 2. Add a step and select the "Transform by Orthology" option. Next select the species you wish to transform your results to (in this case we will select all of them). Click on the Run Step button to get your results.



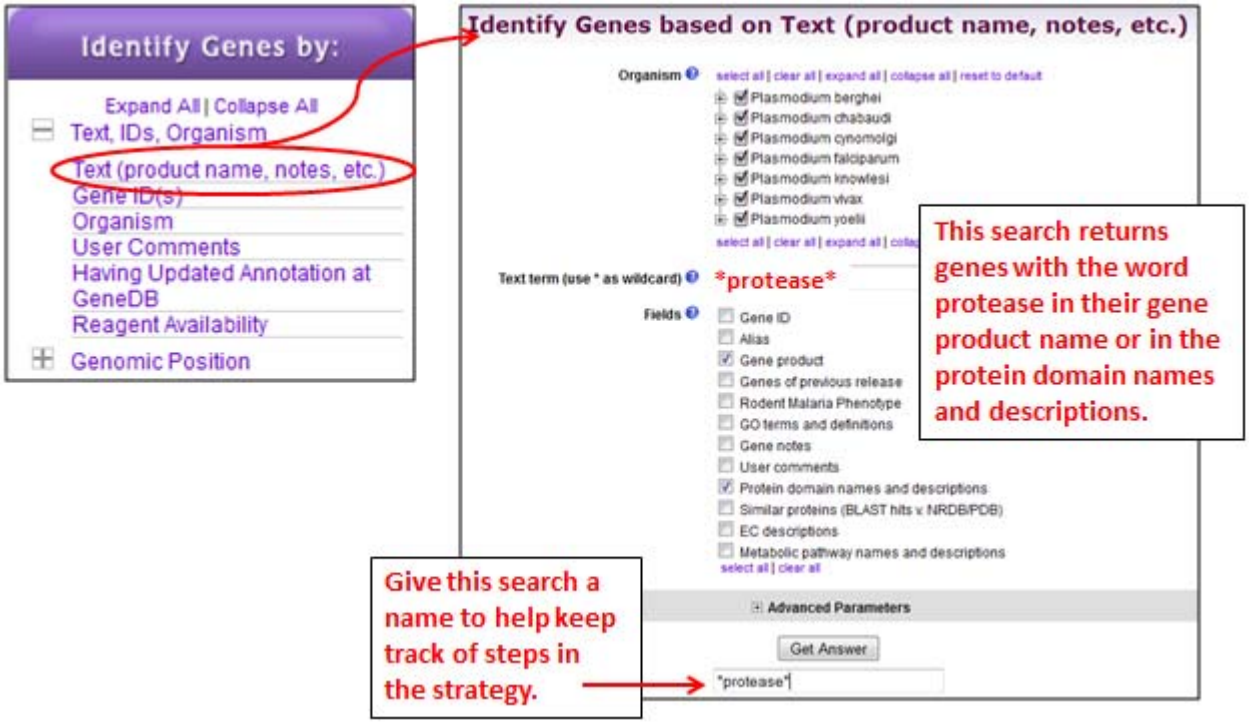
3.2 Examine your results. Note that *P. falciparum* now has 18 genes in the results list, three more that in Example 2 (You can see this in the filter table below the strategy). The increase in the number of *P. falciparum* genes is due to the presence of paralogs.



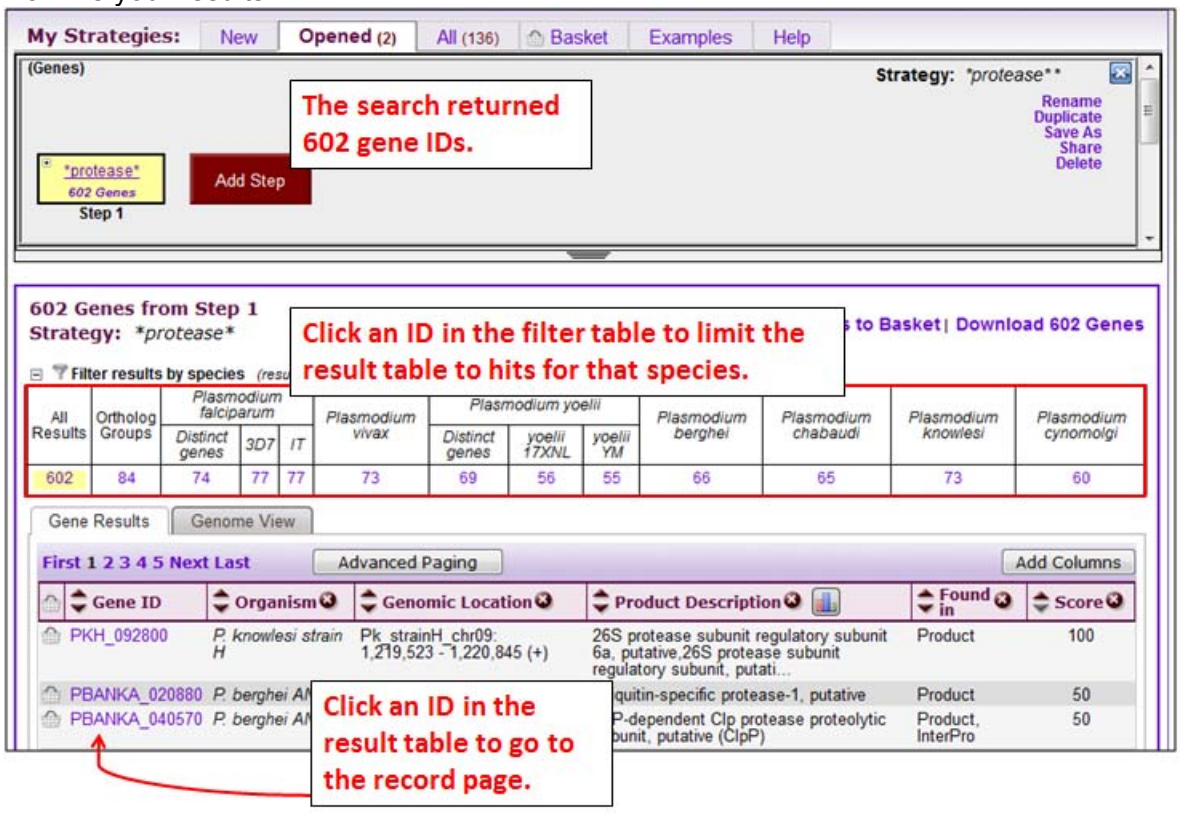
EXAMPLE 4: Find genes whose gene products are possible proteases and contain a motif indicating they may be exported. In this exercise we will build a search strategy that incorporates a nested strategy and download the results for a single species.

4.1 STEP 1 OF YOUR STATEGY. Assemble a list of genes whose protein products may have protease activity.

4.1.1 Run a gene text search using the text term “protease” in the fields “Gene Product” and “Protein domain names and descriptions”. The search “Identify Genes based on Text” returns genes based on three parameters:
Organism: You can choose to limit the search to a subset of annotated genomes. Leaving all organisms in the search is sometimes a good idea since you can use the filter table in the result page to view results from one species.
Text Term: Enter a text term in the box provided. Adding the wildcard symbol (*) before and after the text term will broaden the search to include compound words (ie. metalloprotease) or plurals such as “proteases”.
Fields: There are 12 fields that can be searched for the text term. You can select all but it seems most likely to find proteases if the text term appears in the Gene Product name or the protein description.

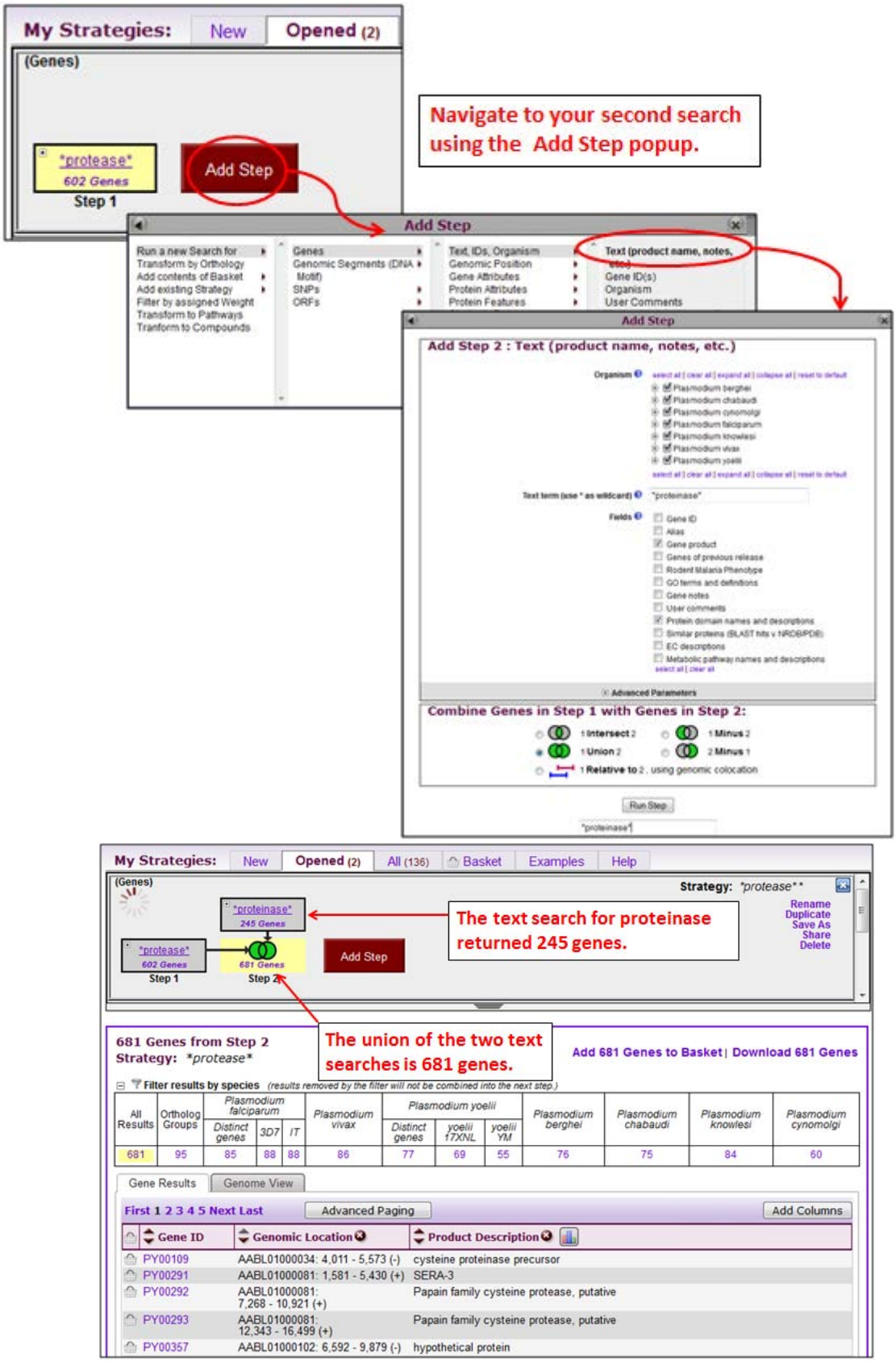


Examine your results:

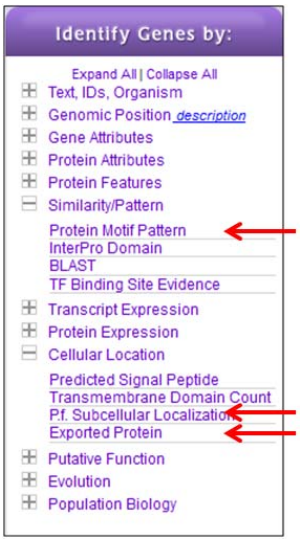


4.2 STEP 2 OF YOUR STRATEGY. Expand your text search to include the text term "proteinase". Add a step to your strategy that duplicates the text search but changes the text term to "proteinase" and join the two search result lists.

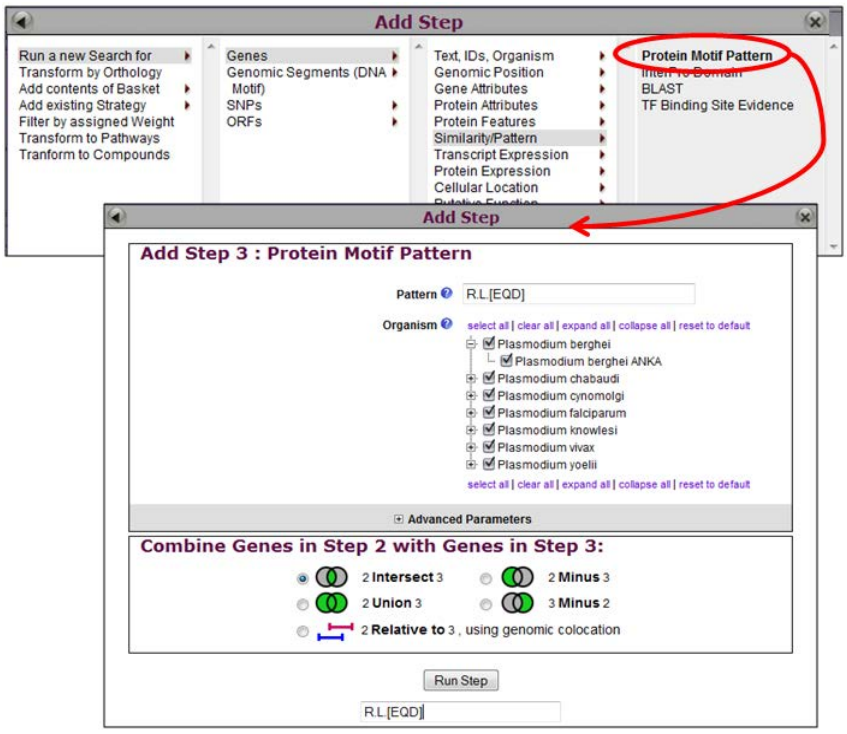
4.2.1Click Add Step and follow the choices in the Add Step popup to reach a second text search page. The "Add Step 2" search form prompts you to define the parameters for a second text search and to define the method for combining searches. Combining searches with "union" will produce a list of genes whose records contain either or both text terms from search and search 2. The result lists from the two searches are combined and duplicates are removed to produce a list of records that are unique across both search result sets.


My Strategies: New Opened (2)
(Genes)
protease
602 Genes
Step 1
Add Step
Navigate to your second search using the Add Step popup.
Add Step
Run a new Search for
Transform by Orthology
Add contents of Basket
Add existing Strategy
Filter by assigned Weight
Transform to Pathways
Tranform to Compounds
Genes
Genomic Segments (DNA Motif)
SNPs
ORFs
Text, IDs, Organism
Genomic Position
Gene Attributes
Protein Attributes
Protein Features
Text (product name, notes, etc.)
Gene ID(s)
Organism
User Comments
Add Step
Add Step 2 : Text (product name, notes, etc.)
Organism
Plasmodium berghei
Plasmodium chabaudi
Plasmodium cynomolgi
Plasmodium falciparum
Plasmodium knowlesi
Plasmodium vivax
Plasmodium yoelii
Text term (use * as wildcard)
"proteinase"
Fields
Gene ID
Alias
Gene product
Genes of previous release
Rodent Malaria Phenotype
GO terms and definitions
Gene notes
User comments
Protein domain names and descriptions
Similar proteins (BLAST hits v NRDB/PDB)
EC descriptions
Metabolic pathway names and descriptions
Advanced Parameters
Combine Genes in Step 1 with Genes in Step 2:
1 Intersect 2
1 Minus 2
1 Union 2
2 Minus 1
1 Relative to 2 , using genomic colocation
Run Step
"proteinase"1
My Strategies: New Opened (2) All (136) Basket Examples Help
(Genes)
Strategy: *protease* *
Rename
Duplicate
Save As
Share
Delete
proteinase
245 Genes
protease
602 Genes
681 Genes
Step 1
Step 2
Add Step
The text search for proteinase returned 245 genes.
The union of the two text searches is 681 genes.
681 Genes from Step 2
Strategy: *protease*
Add 681 Genes to Basket | Download 681 Genes
Filter results by species (results removed by the filter will not be combined into the next step.)
All Results
Ortholog Groups
Plasmodium falciparum
Distinct genes
3D7
IT
Plasmodium vivax
Plasmodium yoelii
Distinct genes
yoelii 17XNL
yoelii YM
Plasmodium berghei
Plasmodium chabaudi
Plasmodium knowlesi
Plasmodium cynomolgi
681 95 85 88 88 86 77 69 55 76 75 84 60
Gene Results
Genome View
First 1 2 3 4 5 Next Last
Advanced Paging
Add Columns
Gene ID
Genomic Location
Product Description
PY00109 AABL01000034: 4,011 - 5,573 (-) cysteine proteinase precursor
PY00291 AABL01000081: 1,581 - 5,430 (+) SERA-3
PY00292 AABL01000081: 7,268 - 10,921 (+) Papain family cysteine protease, putative
PY00293 AABL01000081: 12,343 - 16,499 (+) Papain family cysteine protease, putative
PY00357 AABL01000102: 6,592 - 9,879 (-) hypothetical protein

4.3 STEP 3 OF YOUR STRATEGY: Identify genes that are possibly exported in the red blood cell. PlasmoDB offers several ways to find genes whose protein products are possibly exported. Using the Similarity/Pattern search we can find genes whose protein products contain a motif similar to a PEXEL sequence. Under P.f. Subcellular Localization, PlasmoDB also has specific searches based on published studies that produced algorithms for locating PEXEL motifs, HT motifs, and exported proteins. In this section we will use all these methods to create a list of genes that are possibly exported and then intersect that list with our list of protease genes.

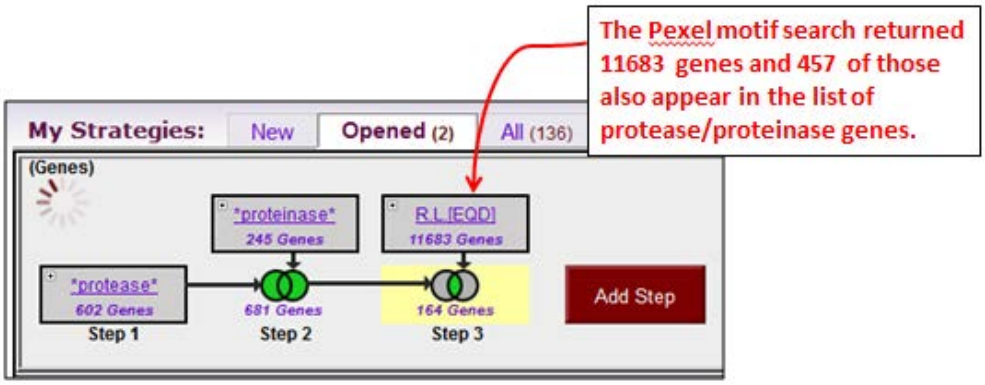


4.3.1 Click Add Step from your strategy and navigate to the search "Identify Genes by Protein Motif Pattern".



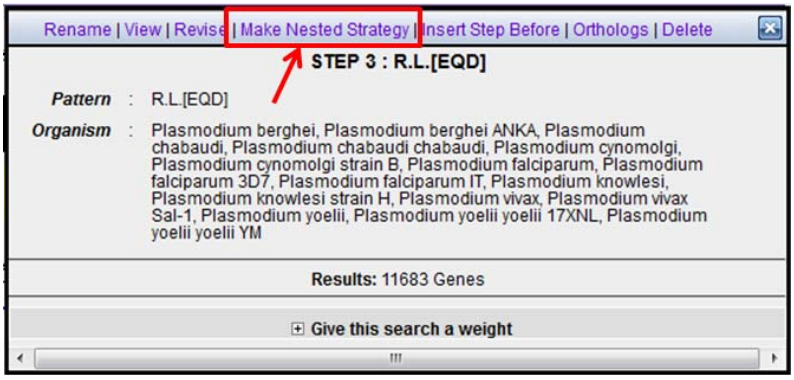
The Protein Motif Pattern Search: A protein motif search scans translated genes for a specific user-defined sequence. The motif can be defined by a simple string of characters representing amino acids or by using a regular expression representing the consensus sequence for the motif of interest. A regular expression for the PEXEL motif will return genes whose predicted protein products contain the motif. Enter a regular expression representing the PEXEL motif (R.L.[EQD]) and make your

choices for the organism parameter. Note that you can give your search a unique name during this step. Choose intersect for the method of combining this new search with your step two results.

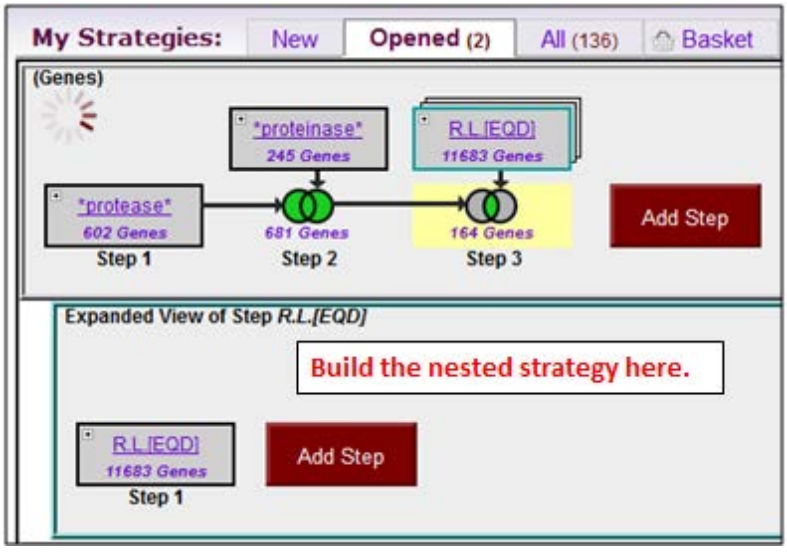


4.3.2 MAKING A NESTED STRATEGY: PlasmoDB has three other searches that specifically look for genes based on the presence of export motifs that were defined in literature references. These searches may return genes that are not in the list of 158 genes, so in the next step we are going to perform more searches and add the list to the protein pattern search results. Click on the Step 3 search name, **PEXEL reg exp**, to revise the Protein Motif Pattern search.

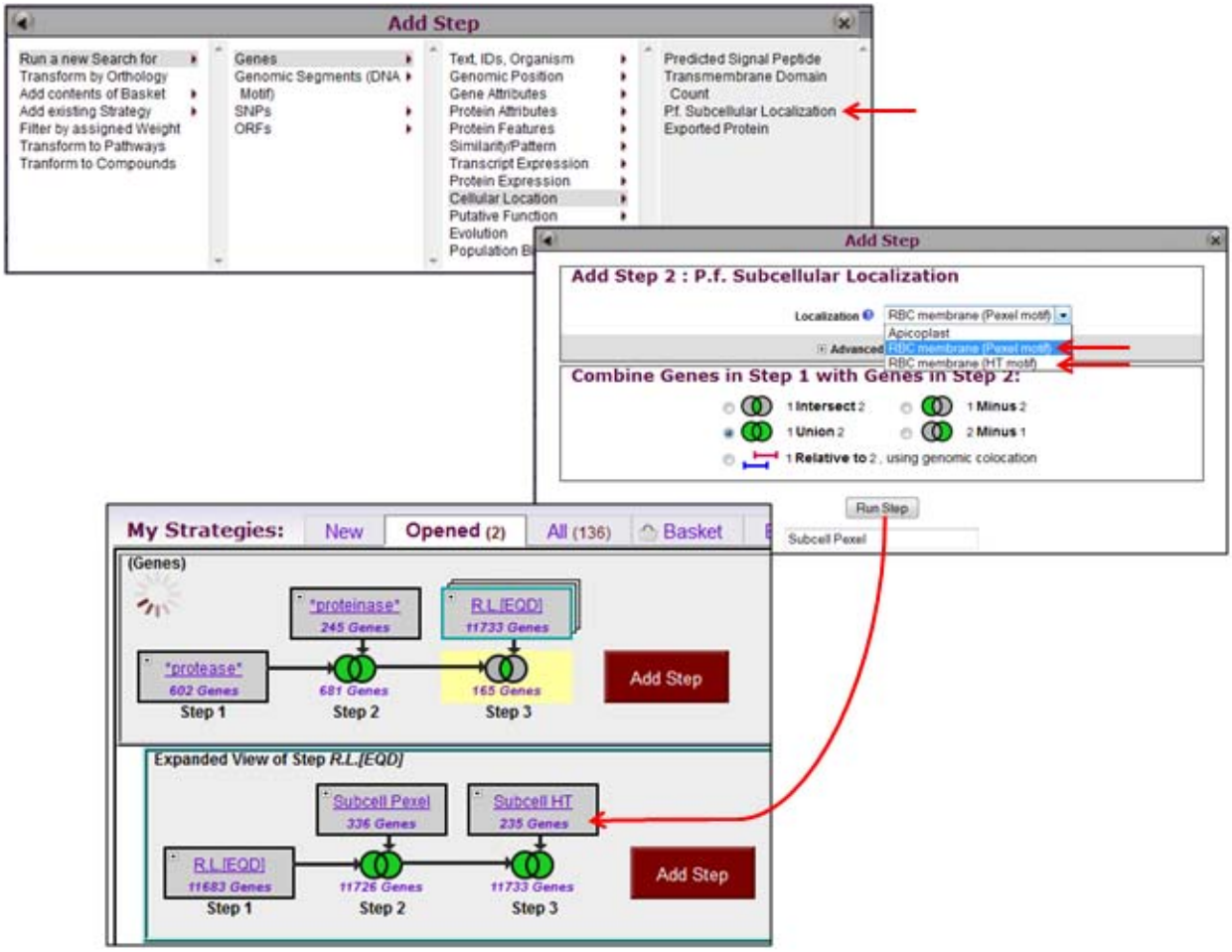
From the Step 3 revise popup window choose "Make Nested Strategy". A new strategy box will open where you can build your nested strategy. A new strategy box will open where you can build your nested strategy.



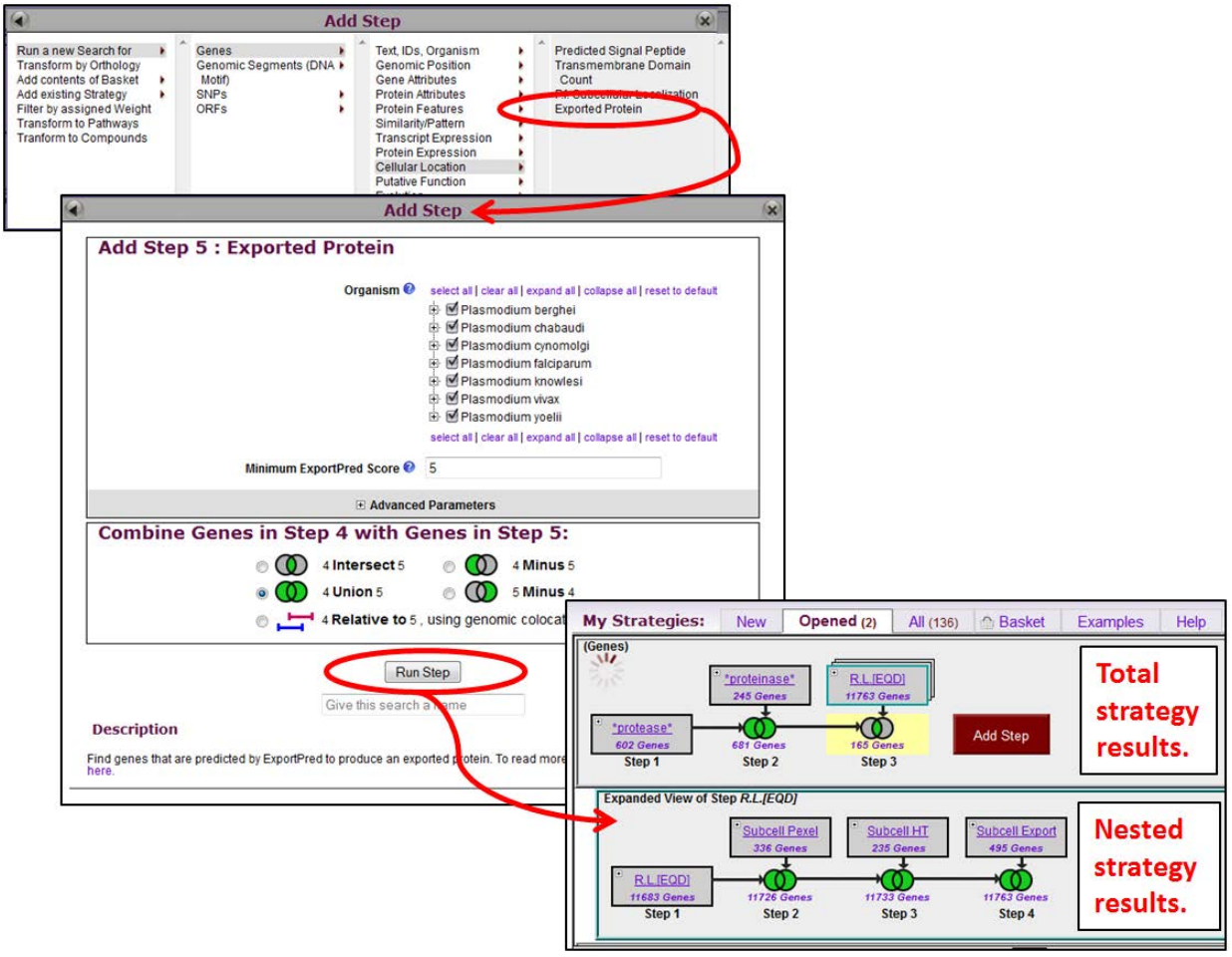
Nesting a strategy allows you to control the logic of the strategy so that, like in an algebraic statement, results are combined in the proper order and the genes in the result list have the correct biological meaning.



4.3.2.1 STEP 2 OF YOUR NESTED STRATEGY: Click Add Step within the nested strategy box and follow the add step popup to choose the P.f. Subcellular Localization search. Choose RBC membrane (Pexel motif), union the search results with the motif search and click run step. The P.f. Subcellular Localization search allows you to search for RBC membrane (Pexel motif) or RBC membrane (HT motif). To maximize the results, run both searches sequentially and combine them using the union operation.



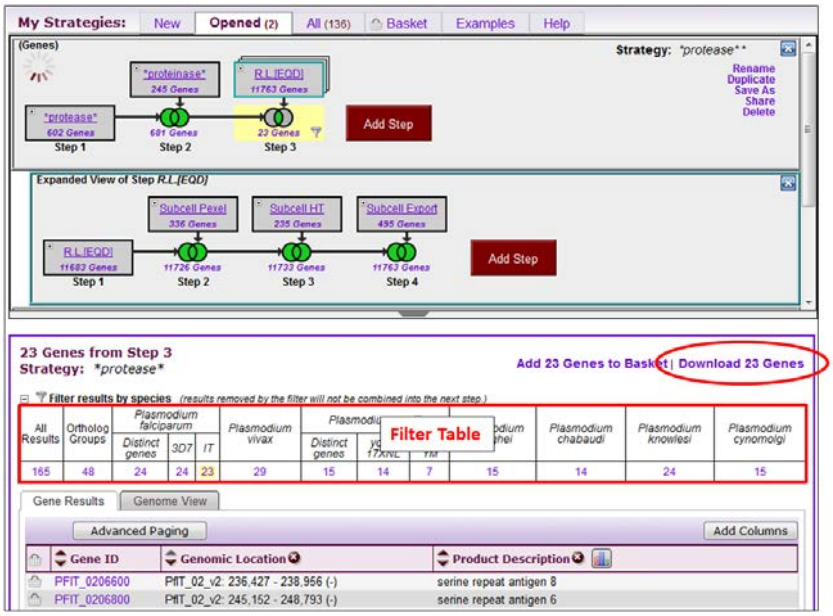
4.3.2.3 STEP 4 OF YOUR NESTED STRATEGY: Run the Exported Proteins search and add the results to your nested strategy. Click Add Step within the nested strategy to include another step. From the Add Step Exported Protein search form, choose the parameters for the search and click Run Step.



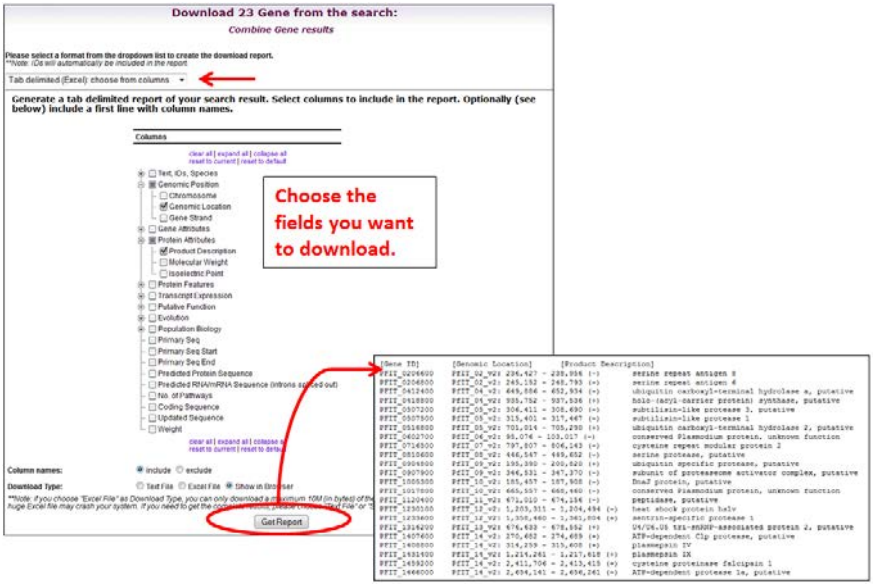
The Subcellular Location Exported Protein search returned 495 genes and increased the nested strategy results by 30 genes.

The total result from the search, the intersection of our protease/proteinase text searches with our nested exported protein search, is 165 genes.

4.4 VIEWING & DOWNLOADING YOUR RESULTS: The result table lists the IDs and other information for the results represented in the yellow highlighted box, in this case, genes whose protein products have been described with the term protease or proteinase and have been identified as bearing motifs that may lead to the export of the protein into a host the red blood cell. You can use the filter table to toggle the result table between species. Click on a number in the filter table to display the subset of results specific to that species. To download the genes that were found in the *P. falciparum* IT genome, first use the filter table to display only the *P. falciparum* IT results, and then click "Download 23 Genes" on the right side of the page above the result table.

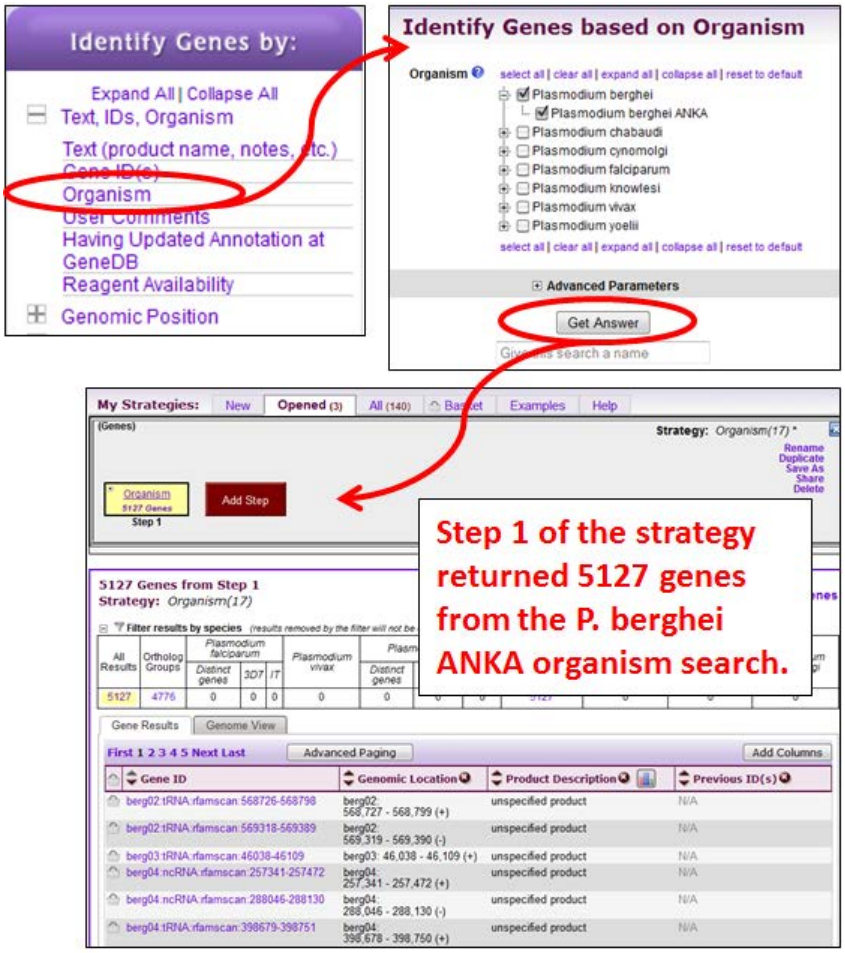


The download page prompts you to choose a file format and the columns of information that you want to download. The record IDs (ie. Gene IDs) are downloaded by default.

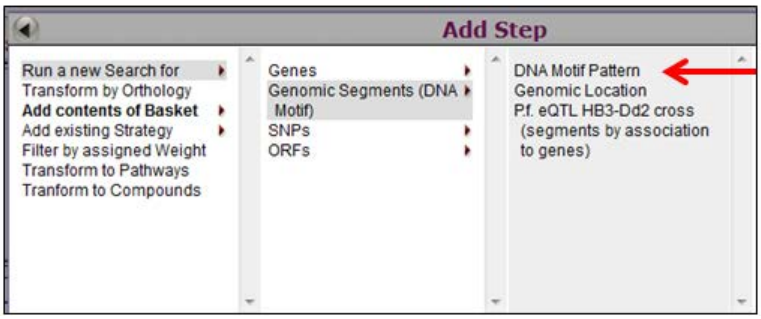


EXAMPLE 5: Searching for DNA motifs in context with the genome. Find all *P. berghei* genes that have AP1 transcription factor binding sites 500 bps upstream of the start site. In this exercise we will first assemble a list of all *P. berghei* genes and then colocate the DNA motif representing the AP1 binding site with the *P. berghei* genes.

5.1 STEP 1 of your strategy: Find all *P. berghei* genes. Use the search "Identify Genes by Text, ID, Organism" to assemble a list of *P. berghei* genes.



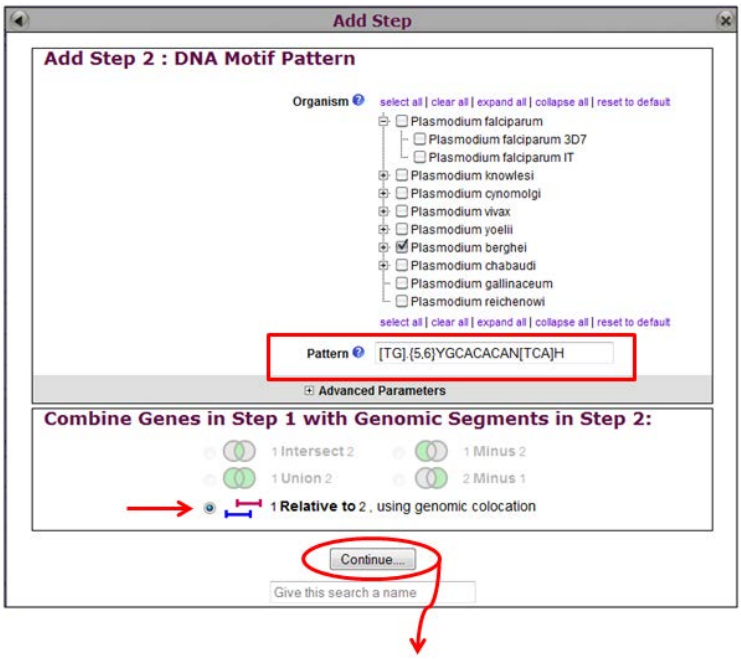
5.2 STEP 2 of your strategy: Search for the consensus sequence of the AP1 transcription factor binding site and combine the returned segments with the *P. berghei* genes using the colocation function.



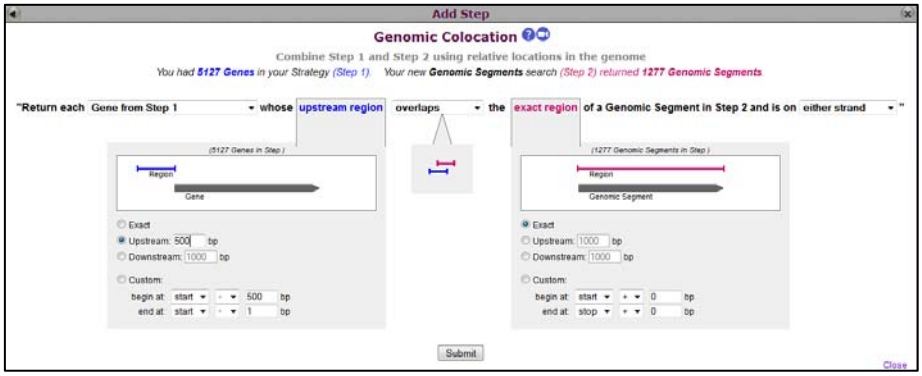
5.2.1 Define parameters for the DNA Motif Pattern search. Click Add Step from the strategy window and follow the choices to the DNA Motif Pattern search.

From the DNA motif pattern search page you will need to define the search parameters and choose a method for combining the genes from the organism search with the segments from the motif search.

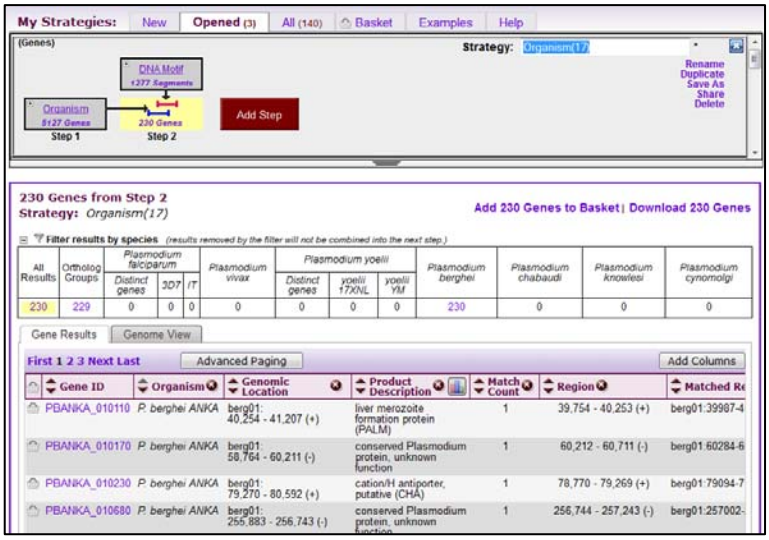
Choose the organism and input the pattern in the text box provided. The default pattern, [TG].{5,6}YGCACACAN[TCA]H, is the regular expression for the AP1 binding site so we can leave that parameter alone.



5.2.2 Choose the method of combining result lists. Choose "1 Relative to 2" to colocate segments to genes. Arrange the logic statement in the Genomic Colocation popup window to return **Genes from Step 1** whose **upstream 500 bp overlaps** the **exact region** of the segment from step 2 and is on **either strand**.

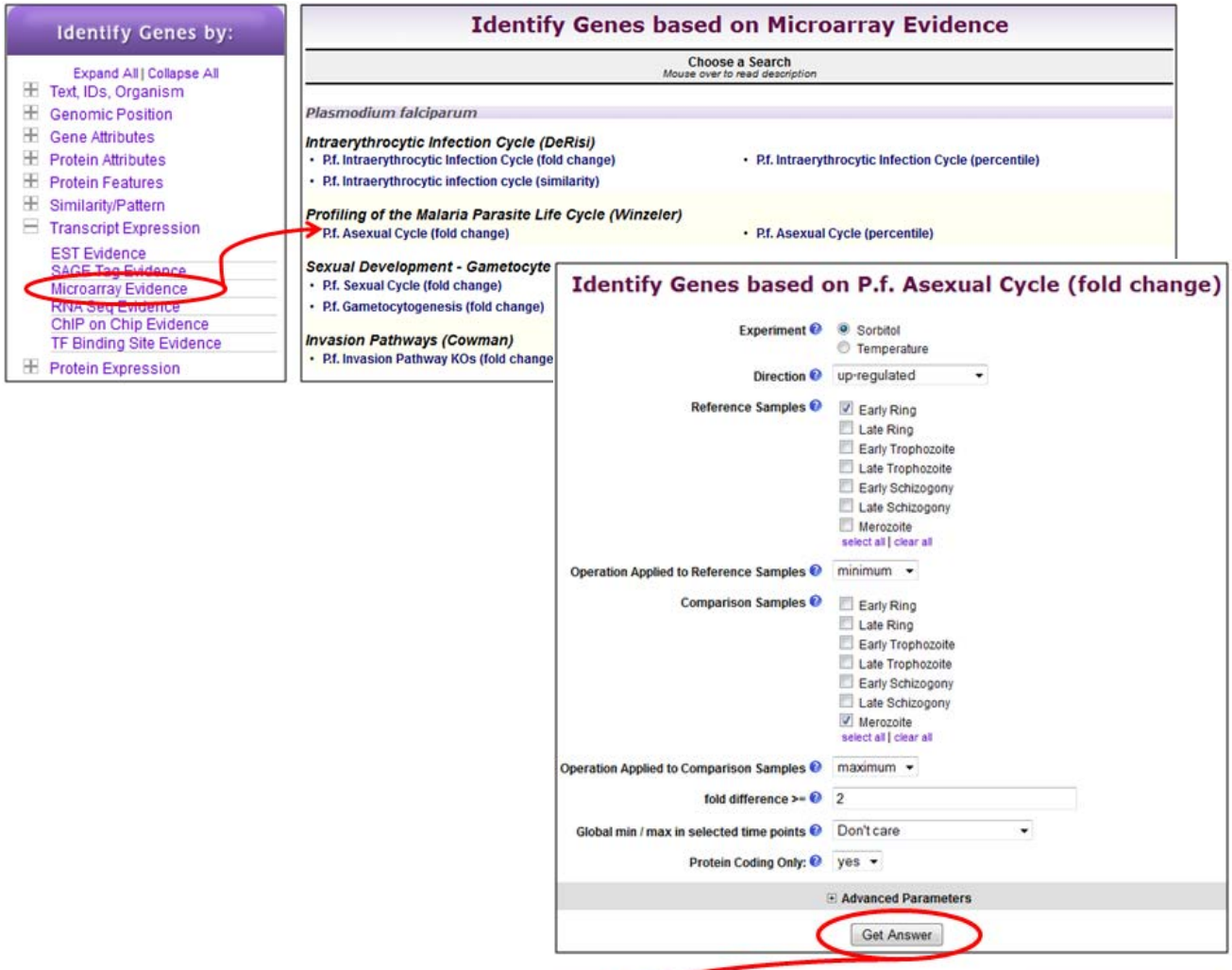


5.3 View your results:
The DNA Motif Pattern search returned 1277 segments that match the consensus sequence. Only 230 *P. berghei* genes contained the motif in the region 500 bp upstream of the start site. The list of genes can be downloaded and each gene record page can be visited by clicking the ID in the result table.

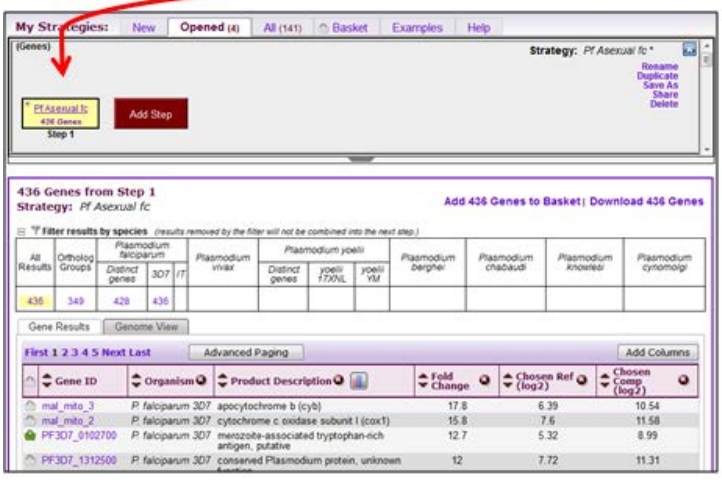


EXAMPLE 6. Find *P. falciparum* genes that are differentially regulated during the asexual life cycle. PlasmoDB includes several transcript expression datasets (ie. microarray, RNA-seq, EST, etc.). This example outlines how one would search data from the microarray study of the *P. falciparum* life cycle (Science. 2003 Sep 12;301(5639):1503-8) for genes that are up-regulated in merozoites compared to the early ring stage.

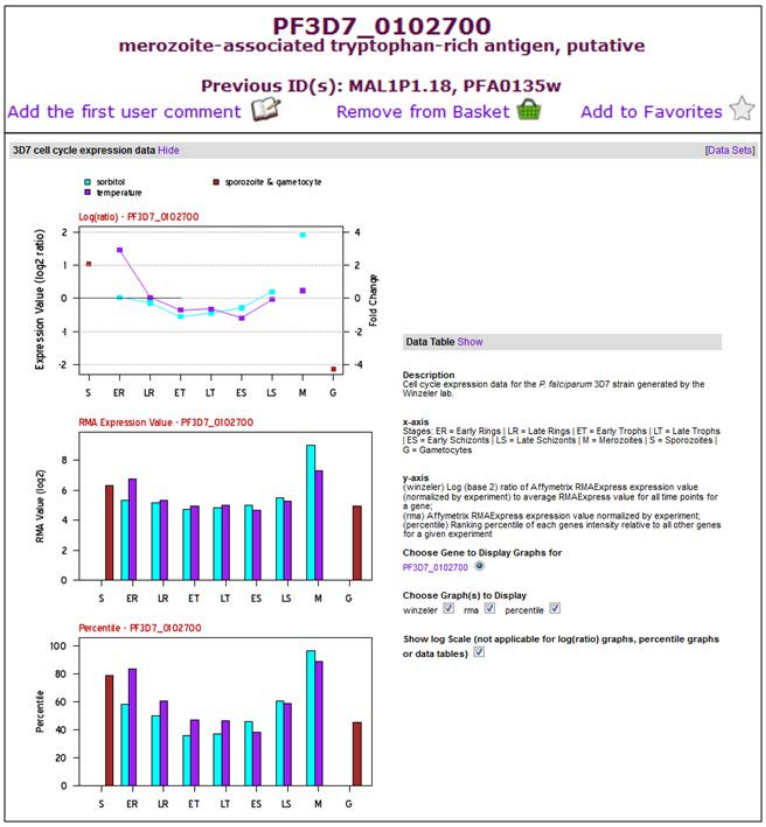
6.1 Run the search: Open the search "Identify Genes based on P.f. Asexual Cycle (fold change)" and define the parameters. A quick look at the literature reference may help in choosing the parameters. For this example, we want to find genes that are up-regulated from Early Ring stage to Merozoites.



6.2 View your results: The search returned 436 genes that, in this study, were found to be up-regulated in merozoites compared to early rings.

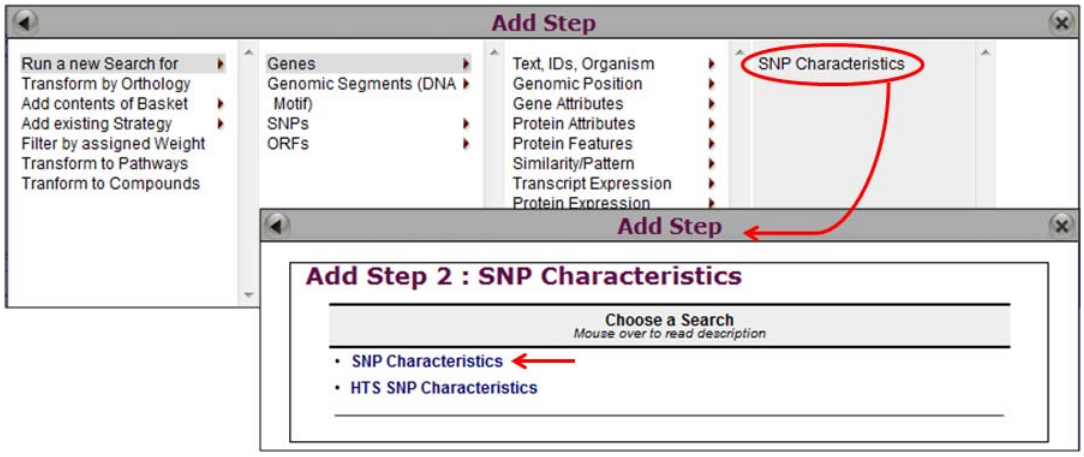


6.3 View microarray data for a specific gene: The subset of microarray data representing an individual gene's expression is displayed on the gene page. From the Expression section of any gene record page you can view the expression data graphed several different ways. Click the ID in the result table to go to the record page. Then scroll down to the expression section and click "SHOW" next to the experiment description to view gene specific microarray results.

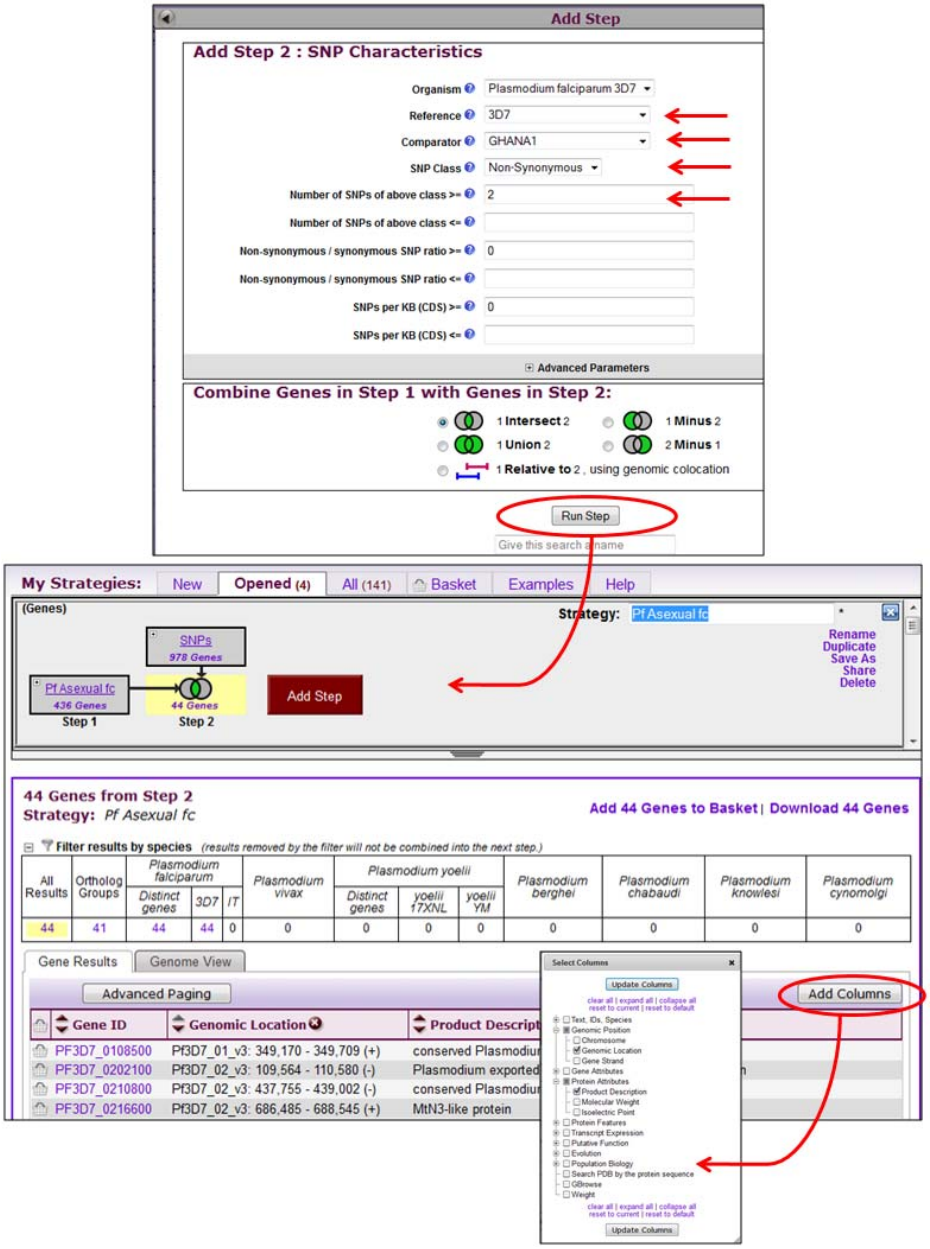


EXAMPLE 7. Using population data to define genes that are under diversifying or purifying selection. Genes in PlasmoDB may be identified based on the number and type of single nucleotide polymorphisms (SNPs) they contain. In this example, find all genes from Example 6 above (genes upregulated in merozoites) that are under diversifying selection (contain an increased number of nonsynonymous SNPs).

7.1 Add a search for genes based on SNP Characteristics: Starting with the search strategy from Example 6, click on "Add Step" and navigate to the SNP Characteristics search.



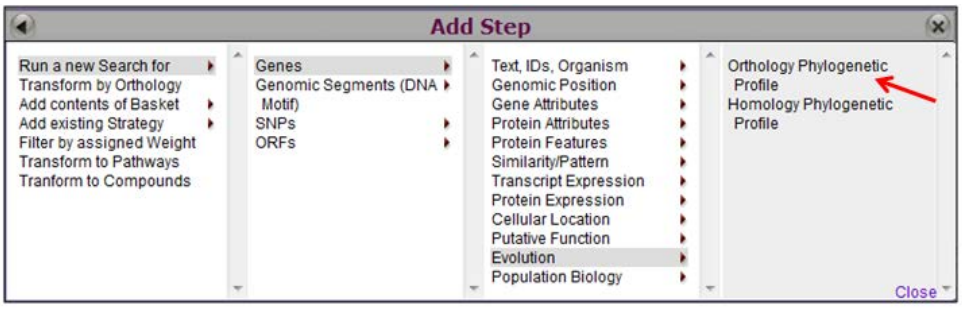
7.2 Choose search parameters: Modify the parameter settings in the SNP characteristics search popup to identify genes that contain at least 2 non-synonymous SNPs between *P. falciparum* 3D7 and the field isolate Ghana1. Intersect the results from this search with those in step 1.



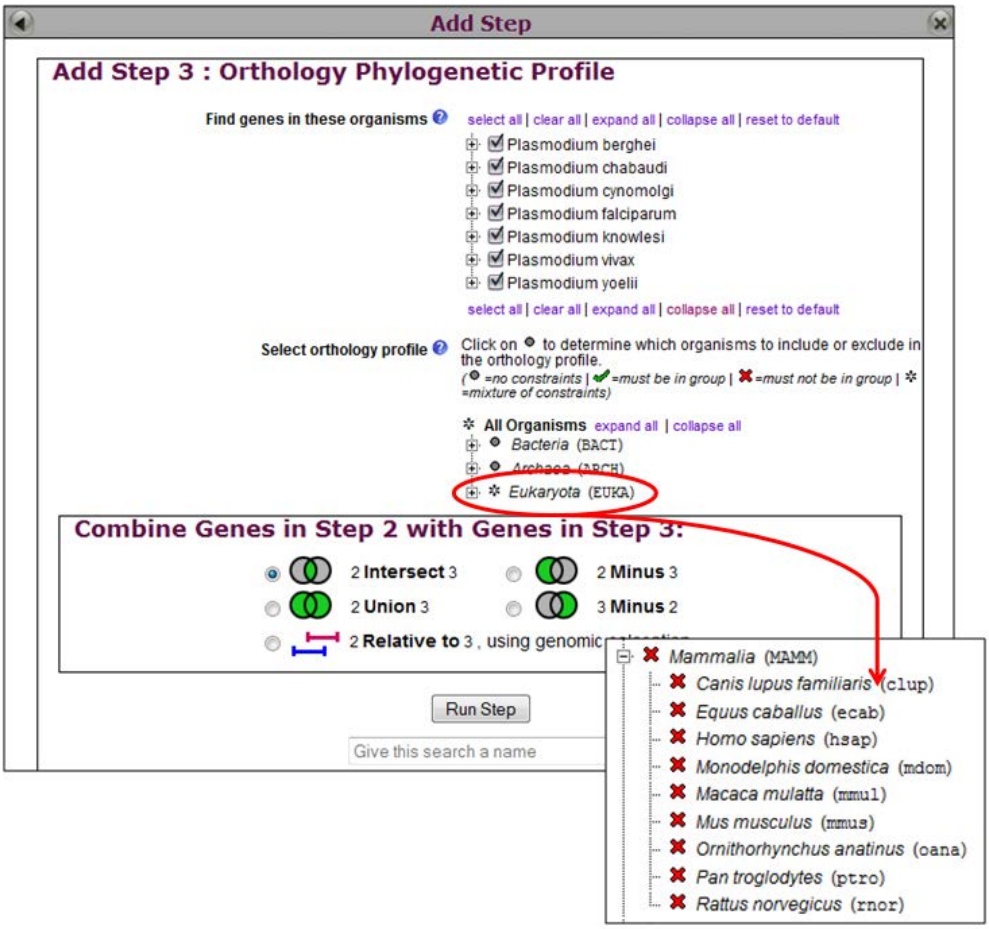
7.3 View the results. To add SNP data columns to your result table Click Add Columns and choose from the Select Columns popup.

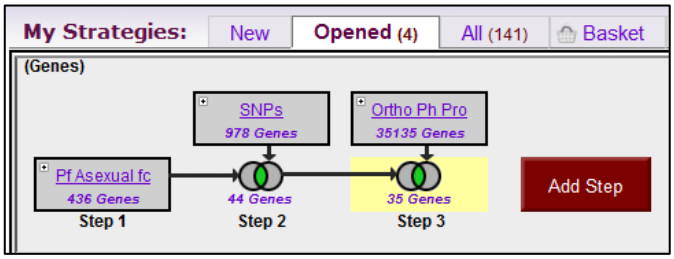
EXAMPLE 8: Leverage orthology to identify *P. falciparum* genes that do not have orthologs in mammals. How many genes form example 7 above are absent from mammals and arguably might be possible vaccine candidates?

8.1 Run a search the Starting with the search strategy from Example 7, click on "Add Step" and navigate to the Orthology Phylogenetic Profile search.



8.2 Choose parameters for the Orthology Phylogenetic Profile search. Configure the orthology phylogenetic profile search form to exclude mammals from the results. A red X (click twice on the gray circle) denotes exclusion. A green check mark (click once on the gray circle) denotes inclusion. Gray circle (no clicking) denotes no restrictions. Click on run step to view your results.





EXAMPLE 9. Complex Strategies: Define possible vaccine candidates in malaria. Find *Plasmodium* genes that could be potential drug targets. There are many ways to do this search; experiment with different parameter settings and incorporating different queries.

a. Considering all of the many 'Queries & Tools' available on PlasmoDB, how many criteria can you define that might be useful for identifying candidate vaccine targets?

b. After ***first*** trying to develop your own query, you might be interested to look at an example query:

Malaria Vaccine Strategy
(http://plasmodb.org/plasmo/im.do?s=14627aa18a8052a5)

Try revising various components of this query to improve it still further to reflect your own insights, theories or experience. ***Note that if you have logged in, you can save the results of your queries for future reference, or to share with others.***

c. How would your results change if you used weighted searches? Revise each step of your strategy and assign a weight to it. The weight is arbitrary; you decide on the scale and the results are sorted based on the sum of the weights. Remember, for weighting to work you have to use the Union operation to join the steps in your strategy. After doing this, what are some of your top candidates? (Hint: you can sort a column in your results by clicking on the arrows next to the column name) Here is the example from above with assigned weights (note that the weighted strategy may take a while to load):

Weighted Vaccine Strategy
(http://plasmodb.org/plasmo/im.do?s=094f2aae5385bbd9)

II. RMgmDB: A genotype and phenotype database of genetically modified rodent malaria parasites

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OVERVIEW

The RMgmDB database (Rodent Malaria parasites Genetically Modified database; www.pberghei.eu) is a manually curated web-based repository that contains information on all published (and some unpublished) genetically modified rodent malaria parasites. It provides easy and rapid access to information on the genotype and phenotype of genetically modified mutant parasites and transgenic (reporter) parasite lines. Here we describe both what is in and how to search the RMgm database. In addition we describe how to submit unpublished information to RMgmDB about mutants which do not exhibit a clear phenotype and on unsuccessful attempts to disrupt or mutate *Plasmodium* genes.

THE DATABASE

RMgmDB contains genotype and phenotype information of mutant and transgenic reporter parasites and information on gene function inferred from the mutant phenotypes (Janse et al., 2011; Khan et al., 2013). Most information has been manually extracted from published scientific literature retrieved from Medline searches. In addition, the database also contains unpublished data on mutants without a clear phenotype and on negative trials to either disrupt or mutate genes. The database is continually updated and, as of March 2013, it contained information on over 780 mutants or unsuccessful attempts to generate mutants. The database can be accessed via appropriate gene-pages in the *Plasmodium* genome resources PlasmoDB (www.plasmodb.org) and GeneDB (www.genedb.org). In PlasmoDB and GeneDB the information of a mutant reported in a rodent *Plasmodium* species is directly linked to the orthologous genes of *P. falciparum* 3D7. In RMgmDB there are direct links to GeneDB (www.genedb.org) and Medline abstracts wherever there is a gene and/or paper(s) associated with the mutant or reporter parasite. In addition to genotype and phenotype data, information is provided on the DNA-constructs used for the genetic modifications. Information on DNA constructs of mutants generated using *Plasmo*GEM vectors is linked to the information provided in the *Plasmo*GEM database (http://plasmogem.sanger.ac.uk/). The database is updated on a weekly basis and collates all the latest data from Medline searches and/or the introduction of unpublished data provided by different laboratories.

The Web Interface

The Web Interface (Figure 1) is designed to search for information on mutant parasites within the database based on searches using either (single or multiple) *Plasmodium* gene models (gene ID's) or using text terms. Moreover, mutants can be searched for using a gene ID or text term in combination with a specific genetic modification. Five different types of genetic modification have been defined: (i) mutants with *disrupted* genes (i.e. 'knock-out mutants'); (ii) mutants where the gene has been *mutated*; (iii) mutants where the gene has been *tagged*; (iv) mutants expressing *transgenes*; and (v) mutants with *'other' modifications* (Figure 1). For mutants that

express *transgenes* the regulatory regions can be further specified in the search field (i.e. the transgene and/or promoter and/or 3' UTR; Figure 1).

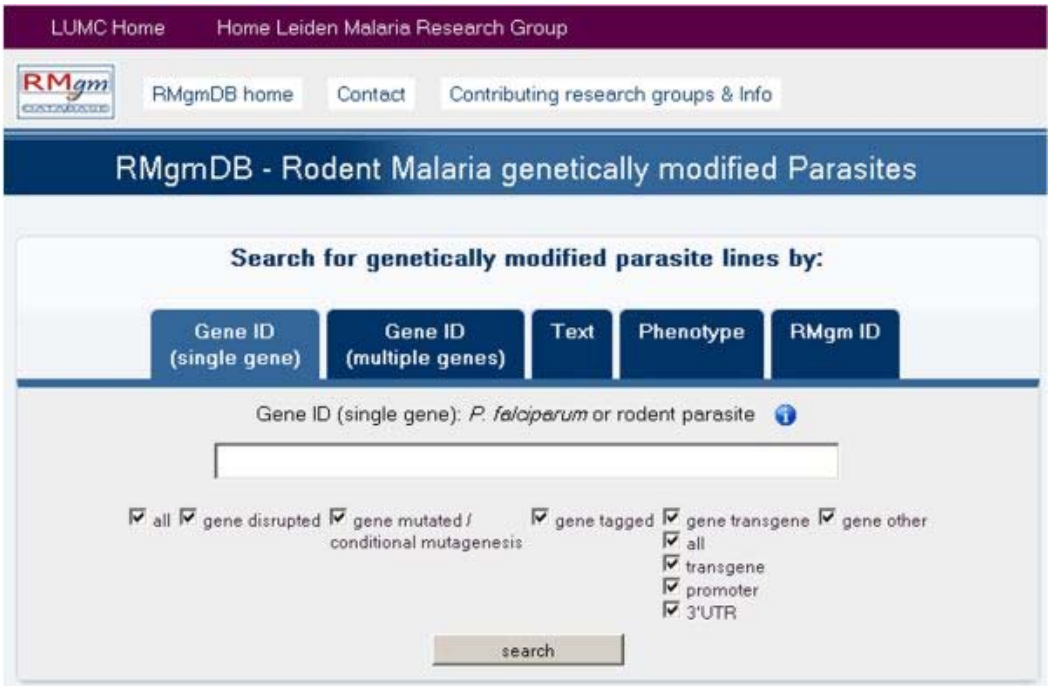

Figure 1: Searching RMgmDB for mutants using single/multiple gene ID's or text terms in combination with one or more types of genetic modification.

In addition to the different genetic modifications, the parasite life-cycle has been divided into six different stages where the phenotype of the mutant is described in comparison to the wild type parasite phenotype (see Figure 2). Specifically, (i) *asexual blood stage* (includes rings trophozoites, schizonts and merozoites); (ii) *gametocyte/gamete* (includes both male and female gametocytes/gametes; (iii) *fertilization and ookinete* (includes zygotes); (iv) *oocyst*; (v) *sporozoite* (includes midgut- and salivary gland sporozoites); and (vi) *liver stage* (includes liver-trophozoite, -schizont and -merozoite). Mutants can be searched for by specifying the life-cycle stage, with a phenotype different from wild-type phenotype, in combination with a specific genetic modification (Figure 2)

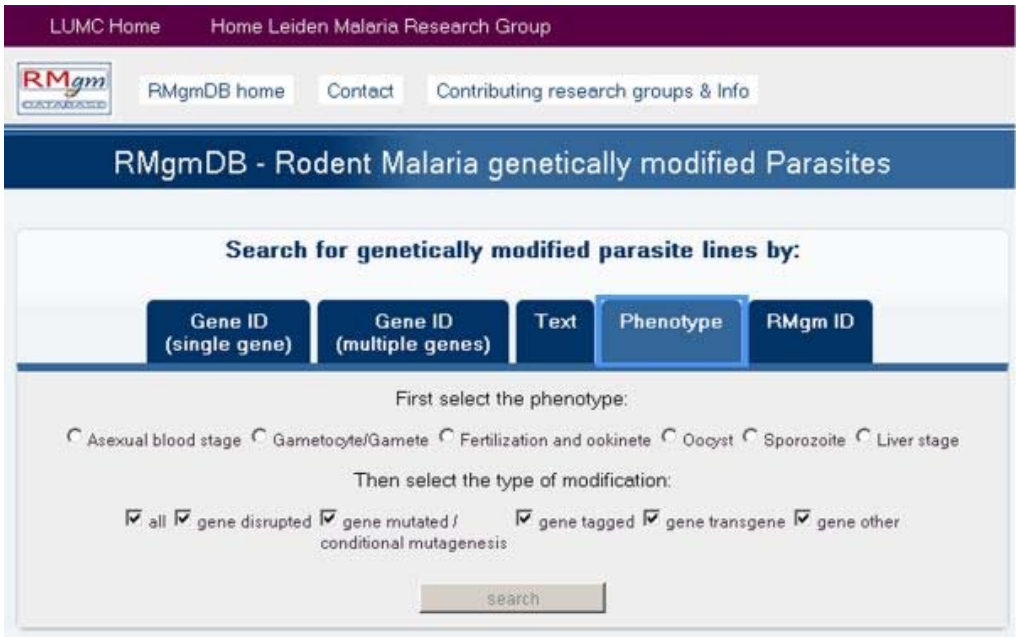

Figure 2: Searching RMgmDB for mutants with a phenotype in one or more life cycle stages which is different from wild type parasites in combination with one or more types of genetic modification.

It is **IMPORTANT** to note that RMgmDB is a database for information on mutant parasites and transgenic reporter parasites. Although it provides information on life-cycle stage mutant phenotypes it does NOT provide a systematically organized set of data on mutant (cellular/molecular) phenotypes or gene-function. The (limited) information in RMgmDB on phenotypes and gene function is provided as 'free text' using the same terminology as used in the publications describing the mutants. The lack of standardized vocabularies in *Plasmodium* for describing phenotypes and gene functions 'inferred from mutant phenotypes' limits the ability to search for mutants with a comparable phenotype or to identify genes with a similar function. For example, it is not possible for a systematic search for all mutants that have a gliding motility phenotype (see example below) or for genes that have a proven role in the motor complex etc. See also Janse et al., (Janse et al., 2011) for a more detailed discussion how the lack of standardized vocabularies for phenotypes and gene functions in *Plasmodium* limits searching RMgmDB and reduces the possibilities of integrating information on gene function and protein location from RMgmDB with gene data from other resources.

Searching the database: Search Results

Searching for mutants using a single gene ID or text terms

Searching the database for specific mutants can be performed by specifying the gene ID in combination with one or more of 5 types of genetic modifications: *disruption, mutation, tagging* and introduction of a *transgene* or *other modification* (Figure 1). Similarly, searching the database for mutants can also be performed using text-terms in combination with one or more of the 5 types of genetic modification (Figure 2; see below for limitations of RMgmDB when searching using text-terms). After submitting your search-query, the search result shows an overview of the mutants or unsuccessful attempts to disrupt or mutate genes (Figure 3; nothing is indicated where either the gene model or text term fail to retrieve any information). The search-results provides a short

summary of the mutant including the type of modification, the gene ID, the gene product and the life cycle stages with a phenotype that is different from the phenotype of wild type parasites. For *mutated* genes a short description of the mutation is given (Figure 3) and for *tagged* genes the name of the tag. For *transgenes* the following information is provided in the search-overview: the name of the transgene, the insertion/replacement locus in the genome, the promoter and the 3'UTR region. Clicking on one of the mutants in the search-results leads to the record page of the mutant. See also below for some examples of searches using a single gene ID or using text terms.

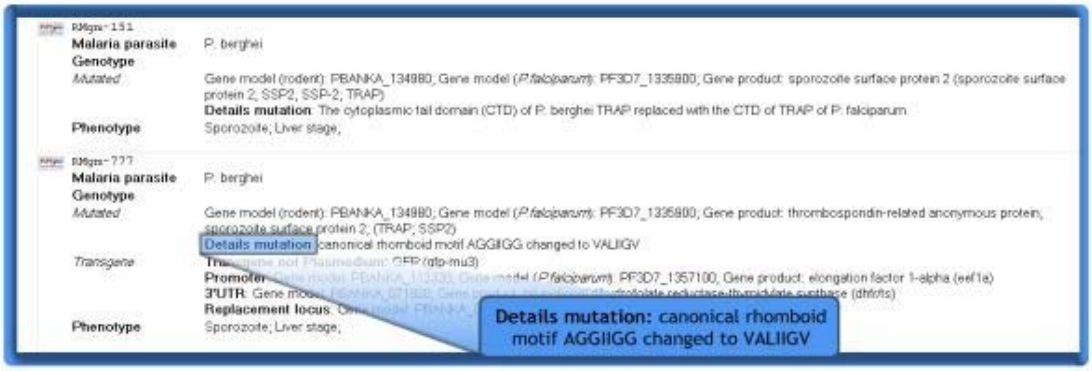

Figure 3: Search results: The search-results provides a short summary of the mutant including the type of modification, the gene ID, the gene product and the life cycle stages with a phenotype that is different from wild type parasites. For *mutated* genes a short description of the mutation is shown (see highlighted box).

Searching for mutants using multiple gene IDs

IMPORTANT: It is possible to 'batch search' for mutants using multiple gene ID's (Figure 4). Consequently, it is possible to retrieve collated information related to multiple genes at once, which not only speeds up searches but also facilitates the cross-linking and mining of data from multiple sources. The output for searches with multiple gene ID's (in table format) include: the gene IDs (rodent and *P. falciparum*); the RMgmDB IDs, the types of modification (*disrupted, mutated, tagged, other modification*); if the modifications were (un)successful; life cycle stages with a phenotype if the phenotype is different from wild type parasites. The search-results from a search using multiple gene IDs is displayed in a table on the website (Figure 5) or it can be downloaded as an Excel/spreadsheet compatible format, i.e. as a CSV file (Figure 4). Clicking on the RMgmDB ID in the table (and spreadsheet) leads directly to the mutant record page.

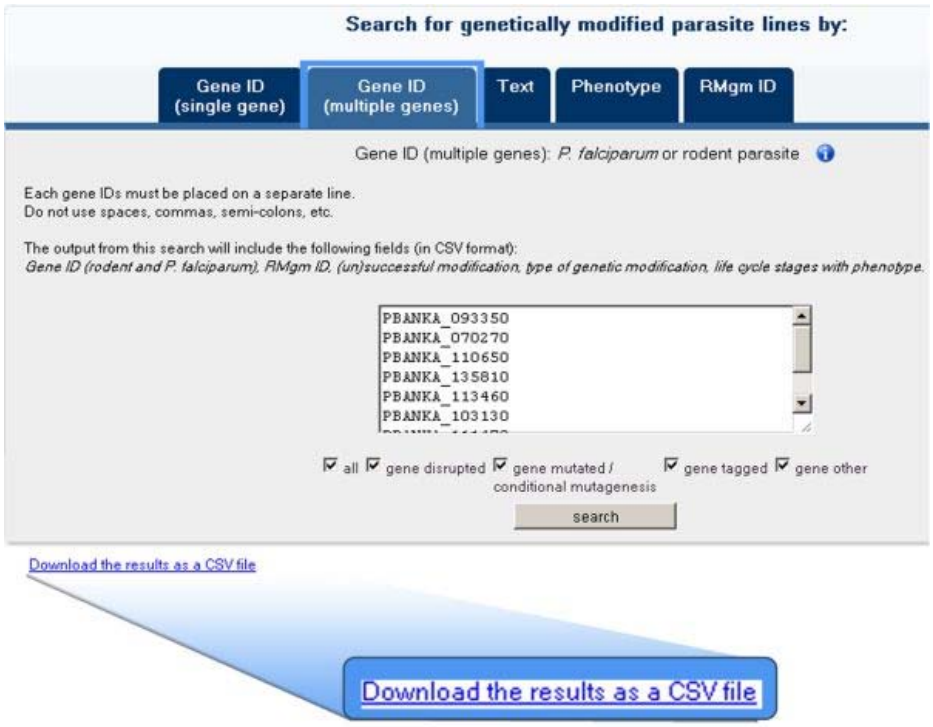

Figure 4: Batch-searching using multiple genes (each gene ID must be placed on a separate line; do not use spaces, commas, semi-colons, etc). If you wish to download the search-results as an Excel/spreadsheet compatible format (i.e. CSV file), click the text indicated otherwise they will be displayed as a table on the website (see Figure 5).

Legends phenotype	
X	Phenotype different from wild type
nd	Phenotype not different from wild type
nt	Not tested

					Modification type				Phenotype					
Searched ID	RMgmDB	Gene ID rodent	Gene ID falciparum	Successful modification	Disrupted	Mutated	Tagged	Other	Asexual blood stage	Gametocyte, Gamete	Fertilization and ookinete	Oocyst	Sporozoite	Liver stage
PBANKA_093350	RMgm-176	PBANKA_093350	PF3D7_1114100	yes	X				X	nd	X	X	X	X
PBANKA_093350	RMgm-177	PBANKA_093350	PF3D7_1114100	yes	X				nd	nd	nd	nd	nd	X
PBANKA_093350	RMgm-761	PBANKA_093350	PF3D7_1114100	yes	X				nd	nd	nd	nd	nd	X
PBANKA_070270	RMgm-178	PBANKA_070270	PF3D7_0828000	yes	X				nd	nd	nd	X	X	nt
PBANKA_070270	RMgm-763	PBANKA_070270	PF3D7_0828000	yes			X		nd	X	X	X	nt	nt
PBANKA_110650	RMgm-107	PBANKA_110650	PF3D7_0506900	no	X									
PBANKA_110650	RMgm-764	PBANKA_110650	PF3D7_0506900	yes			X		X	X	nt	nt	nt	nt
PBANKA_135810	RMgm-758	PBANKA_135810	PF3D7_1345200	no	X									
PBANKA_113460	RMgm-759	PBANKA_113460	PF3D7_1358300	no	X									
PBANKA_103130	RMgm-760	PBANKA_103130	PF3D7_1411200	no	X									
PBANKA_111470	RMgm-762	PBANKA_111470	PF3D7_0515100	yes	X				nd	nd	nd	nd	nd	nd
PBANKA_111780	RMgm-179	PBANKA_111780	PF3D7_0618600	yes	X				nd	nd	nd	nd	nd	nd

Figure 5: Example of a search results when using multiple gene IDs, displayed in a table on the website.

Searching with multiple gene ID's will, for example, allow the user to (i) rapidly identify genes that have been targeted for disruption but did not result in selection of mutants, indicating an essential role for these genes in blood stage development; (ii) identify genes that give a phenotype at multiple life cycle stages, such as asexual blood stages, gametocytes/gametes, oocysts, liver stages etc.; (iii) identify genes that have been tagged (for example with fluorescent markers); and (iv) identify genes for which no information based on mutant phenotypes currently exists. See also below for some examples of searches using multiple gene ID's.

Searching for mutants with a phenotype in (a) specific life cycle stage(s)

The database can be searched for mutants with a phenotype in one or more life cycle stage if the phenotype is different from wild type parasites (Figure 2). The following life cycle stages can be specified in combination with one or more of the 5 types of modification: *asexual blood stage, gametocyte/gamete, fertilization and ookinete,* oocyst, *sporozoite* and *liver stag*e. See below for an example of a search for mutants with a phenotype at (a) specific life cycle stage(s).

The mutant Record Page

Clicking on one of the mutants in the search-overview, as described above, leads to the record page of the mutant. The information within the database for each mutant parasite has been subdivided into three sections (Figure 6).

The first section provides general information on the generation of the mutant, which includes links to the relevant Medline publications describing the mutant and information on the researchers and research group who generated the mutant.

The second section provides information on the procedures used to generate and select the mutants, and information on the genotype of the mutants. Five different types of genetic modification have been defined: *disruption*, *mutation*, *tagging, transgene expression* and '*other modifications*'. A single mutant parasite may contain multiple different genetic modifications. For each modification, details are provided of the targeted gene, such as gene ID which is linked to GeneDB and the 'gene product name' as provided by GeneDB.

- Mutants with altered genes designed to conditionally regulate gene expression fall in the category *mutated genes*. For example, mutants with 'flirted' genes used to conditionally remove/silence genes using the FLP/FRT recombinase system (Lacroix et al., 2011), mutants with modified/substituted promoters (i.e. 'promoter swap mutants') used to silence gene expression at specific stages of development, e.g. gametocytes or ookinetes (Laurentino et al., 2011; Siden-Kiamos et al., 2011; Sebastian et al., 2012), or mutants with modified genes that contain a tet-inducible promoter for down-regulation of expression by tetracycline derivatives (Pino et al., 2012).
- Genes that have been replaced with an ortholog from another *Plasmodium* species or from another organism are classified as *mutated genes*.
- However, if orthologs are introduced as an additional copy either as episomal plasmids or integrated into a 'silent' locus of the genome, the orthologs are classified as *transgenes*.
- Genes encoding reporter proteins such as fluorescent and luminescent proteins are classified as *transgenes*.
- The database contains a large number of mutants containing epitope-tagged proteins, tagged with for example fluorescent proteins or c-Myc.

- The category '*other modifications*' include for example mutants containing a transposase-mediated *piggyBac* insertion in their genome (Fonager et al., 2011), mutants containing circular or linear '*Plasmodium* artificial chromosomes' (C-PAC; L-PAC) (Iwanaga et al., 2010; Iwanaga et al., 2012a; Iwanaga et al., 2012b)or GIMO-transfection reference mutants that contains the *hdhfr::yfcu* positive-negative selection marker in the silent *230p* locus (Lin et al., 2011).
- For all *mutated genes* a detailed description of the mutation exists and, in addition, a short description of what type of mutation has been engineered is reported in the overview of search-results (Figure 3).

Guidelines for generation, genotyping and describing/reporting of rodent malaria parasite mutants have been provided and discussed in Janse *et al.* and Khan *et al.* (Janse et al., 2011; Khan et al., 2013).

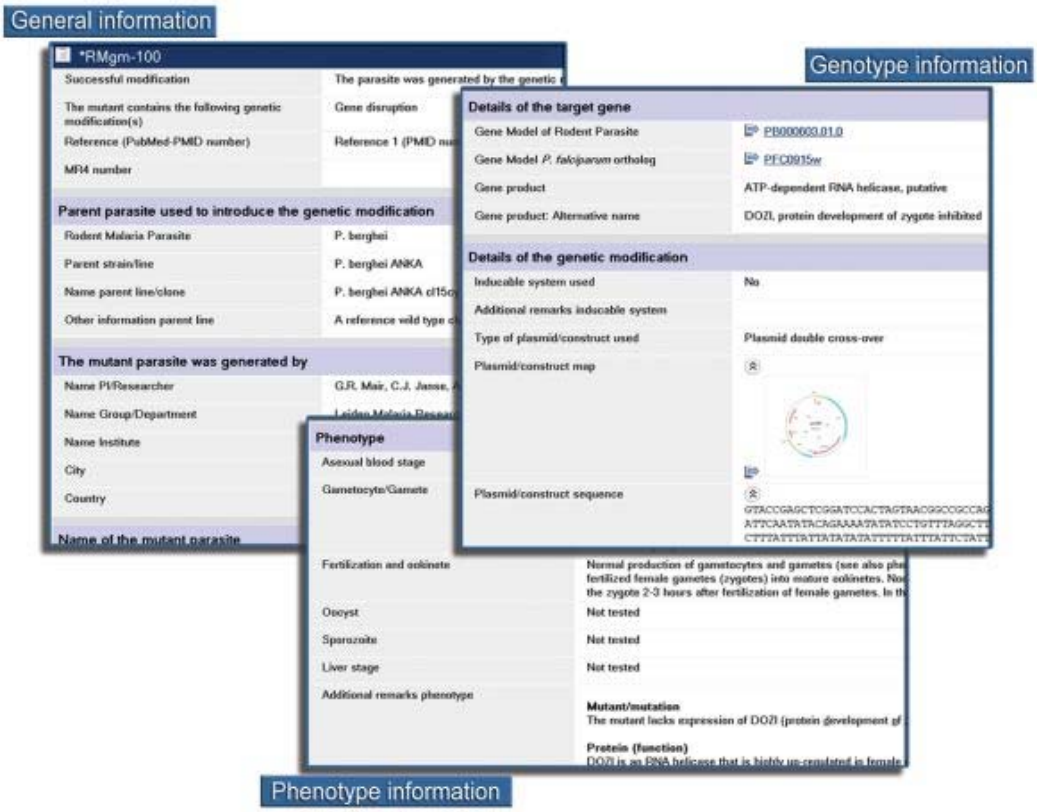

Figure 6: The record page of all mutants: General information and details on genotype and phenotype are provided for each mutant parasite (Janse et al., 2011)

The third section of the mutant information consists of phenotype descriptions of the mutants as well as some additional information on the (function of) gene/protein that has been targeted by the genetic modification. The phenotype description is subdivided according to different parasite life-cycle stages. The six different life cycle stages are: *asexual blood stage, gametocyte/gamete*, *fertilization and ookinete, oocyst, sporozoite* and *liver stage*. Together with the phenotype description, information is provided on the gene function that is inferred from the phenotype analyses and from additional assays described in the publications.

RMgmDB also contains information on unsuccessful attempts to disrupt or mutate genes, and therefore these genes may be refractory to mutation or the mutation may be lethal for asexual blood stage growth. For these unsuccessful trials, we provide information on the DNA constructs used in modifying the genes, the selection procedure and the number of independent transfections attempted.

Examples of searches

1) Search the database for mutants with a disrupted or mutated gene encoding TRAP (thrombospondin-related anonymous protein; gene ID PF3D7_1335900).

- Select 'gene ID (single gene)' tab (Figure 1)
- Type/paste in the search box the gene ID, PF3D7_1335900 (**IMPORTANT**: using the *P. falciparum* Gene ID will retrieve corresponding mutants in all rodent *Plasmodium* species, however if you search with the *P. berghei* gene ID, the corresponding *P. yoelii* mutants will NOT appear in the search-overview)
- Specify the type of modification by selecting 'gene disrupted' and 'gene mutated/conditional mutagenesis' fields (Figure 1)

This search (as of March 2013) results in 18 different mutants, 16 of which contain a mutated *trap* gene**. IMPORTANT**: In the search-overview a short description is given of the type of mutation; for example; 'the canonical rhomboid motif AGGIIGG changed to FFFIIGG' (highlighted in Figure 3). Clicking on one of the mutants will show the record page with the genotype and phenotype of this specific mutant.

2) Search the database for all mutants for the term 'gliding motility'

- Select 'Text' tab (Figure 1)
- Type/paste in the box the text term 'gliding motility'
- Specify all 5 types of modification by selecting 'all' (Figure 1)

This search (as of March 2013) retrieves and displays 87 mutants. **IMPORTANT**: Since text terms search the complete database, and the 'gliding motility' phenotype has not been defined as a specific feature, this search does **NOT** provide a list of only the mutants with a gliding motility phenotype that is different from wild type. For example, mutants appear in this list where in the phenotype description is mentioned that '*the disruption/mutation/tagging has no effect on gliding motility*'. As mentioned above the design of RMgmDB and 'free text' searching, combined with the lack of defined vocabularies for *Plasmodium* phenotypes or gene function, limits the use of text terms searches to identify mutants with defined cellular or molecular phenotypes or to identify genes with a defined function that can be inferred from mutant phenotypes.

3) Search the database for disrupted or mutated gene mutants that have a sporozoite phenotype that is different from wild type sporozoites

- Select 'Phenotype' tab (Figure 2)
- Select 'Sporozoite' field
- Specify the type of modification by selecting gene 'disrupted' and 'gene mutated/conditional mutagenesis' (Figure 2)

This search (as of March 2013) retrieves 140 mutants. **IMPORTANT**: This search will also include mutants that have an 'oocyst phenotype' resulting in reduced or absent production of sporozoites. Therefore, when searching mutants with a specific 'life cycle phenotype' as defined in RMgmDB it cannot be concluded that all mutants in the search-overview have a molecular/cellular function only in that specific life-cycle stage.

4) Search the database for all mutants with disrupted or mutated *Plasmodium* protein kinase genes

- Select 'Gene ID (multiple genes)' tab (Figure 4)
- Type/paste in the box all gene ID's of *P. falciparum* or rodent *Plasmodium* protein kinases (each gene ID must be placed on a separate line; do not use spaces, commas, semi-colons, etc.). **IMPORTANT**: using the *P. falciparum* Gene IDs will retrieve mutants made in all rodent *Plasmodium* species; however if the *P. berghei* gene IDs are used the *P. yoelii* mutants will not appear in the search-overview
- Specify the type of modification by selecting 'gene mutated/conditional mutagenesis' and 'gene tagged' (Figure 4)

The output from this search is displayed in a table on the website (Figure 5) or it can be downloaded as an Excel/spreadsheet compatible format (i.e. as a CSV file; Figure 4). The table and excel sheet include the following fields: Gene ID (rodent and *P. falciparum)*, RMgmDB ID, successful modification, type of genetic modification, life cycle stages with a phenotype that is different from wild type parasites.
This search (as of March 2013) retrieves a search-overview of more than 50 kinase genes that were refractory to disruption/mutation; 18 genes that were amenable to disruption/mutation and the mutants exhibit phenotypes at various points of the life-cycle, of these 5 have a phenotype during fertilisation/zygote stage.

5) Search the database for all mutants which express a tagged *Plasmodium* rhomboid protease

- Select 'Gene ID (multiple genes)' tab (Figure 4)
- Type/paste in the box all gene IDs of *P. falciparum* or rodent *Plasmodium* of the 8 rhomboid protease encoding genes (each gene ID must be placed on a separate line; do not use spaces, commas, semi-colons, etc.)
- Specify the type of modification by selecting 'all' (Figure 4).

This search (as of March 2013) retrieves in a search-overview all mutants that contain mutated, disrupted or tagged rhomboid encoding genes (Figure 5), including 2 rhomboids tagged with GFP or mCherry (ROM3, PBANKA_070270, RMgmDB-763 and ROM4, PBANKA_110650, RMgmDB-764). Note: Selecting 'all' generates a table with all mutants that contain genetically modified rhomboid genes including the tagged rhomboids (Figure 5). Selecting 'tagged' will only show the mutants where the *Plasmodium* rhomboid protein has been tagged.

Submitting information on unpublished mutants without a clear phenotype or negative attempts to disrupt/mutate genes to RMgmDB

RMgmDB is intended to be a dynamic and responsive resource, and researchers are encouraged to update the information on phenotypes of the mutants they have generated. Importantly, much unpublished data exist on *Plasmodium* mutants that were generated but did not show a clear phenotype or where researchers attempted, on multiple occasions, to disrupt a parasite gene but were unsuccessful. This information, while difficult to publish, is of significant value and can also prevent an unnecessary duplication of effort. Moreover, the existence of null-mutants without a distinct phenotype might provide information about the functional redundancy of the target gene. Similarly, the lack of an observable phenotype might also be the result of assays that are currently inadequate or, as yet, too insensitive to reveal a phenotypic effect of the genetic modification. Further analysis of such mutants in improved phenotype assays might

reveal novel aspects of gene function. Below is shown the template for submitting to RMgmDB the information on unpublished mutants and negative trials to disrupt or mutate genes.

Submission of information on mutants already available in RMgmDB

For all mutants a 'comment box' is available on the mutant record page where additional information can be submitted: corrections, comments, or suggestions for improving the description of the mutants.

Submitting information to RMgmDB on unpublished mutants without a clear phenotype

Information on new mutants without a clear phenotype can be submitted in spread sheet (Excel) format. This must include a minimum set of information on the constructs used and the mutant, see Table 1 (left-hand column).

Submitting information to RMgmDB on negative attempts to disrupt or mutate genes

Information on negative attempts to disrupt or mutate genes can be submitted in spread sheet (Excel) format. This must include a minimum set of information on the constructs used and number of attempts to disrupt the gene of interest, see Table 1 (right-hand column).

Table 1
Information required by RMgmDB to report unpublished mutants without a clear phenotype (left-hand column) or unsuccessful attempts to disrupt/ mutate genes (right-hand column). Adapted from (Khan et al., 2013).

Mutants without a clear phenotype	**Unsuccessful attempts to disrupt/mutate genes**
- Rodent parasite species - Parent strain/isolate/line - Name PI/Researcher - Name Group/Department - Name Institute	- Rodent parasite species - Parent strain/isolate/line - Name PI/Researcher - Name Group/Department - Name Institute
- Gene Model of Rodent Parasite (as cited in GeneDB) - Type of modification: disruption/mutation - Name of mutant - Plasmid/construct - double or single cross-over - Partial or complete disruption ORF - Other details of mutation, if applicable - Selection method (drug-treatment, FACS, etc.) - Selectable marker - Promoter selectable marker - Drug used to select	- Gene Model of Rodent Parasite (as cited in GeneDB) - Type of modification: disruption/mutation - Not applicable - Plasmid/construct - double or single cross-over - Partial or complete disruption ORF - Other details of mutation, if applicable - Selection method (drug-treatment, FACS, etc.) - Selectable marker - Promoter selectable marker - Drug used to select
- Primer information (= sequence and name) *Primers for disruption* primer sequence target region 5' forward primer sequence target region 5' reverse primer sequence target region 3' forward primer sequence target region 3' reverse *Other primers used for gene mutation*	- Primer information (= sequence and name) *Primers for disruption* primer sequence target region 5' forward primer sequence target region 5' reverse primer sequence target region 3' forward primer sequence target region 3' reverse *Other primers used for gene mutation*
- Mutant cloned? (yes or no) - Not applicable.	- Not applicable - Number of transfection attempts

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References
Fonager,J., Franke-Fayard,B.M., Adams,J.H., Ramesar,J., Klop,O., Khan,S.M., Janse,C.J., and Waters,A.P. (2011). Development of the piggyBac transposable system for *Plasmodium berghei* and its application for random mutagenesis in malaria parasites. BMC. Genomics *12*, 155.

Iwanaga,S., Kaneko,I., and Yuda,M. (2012a). A high-coverage artificial chromosome library for the genome-wide screening of drug-resistance genes in malaria parasites. Genome Res. *22*, 985-992.

Iwanaga,S., Kato,T., Kaneko,I., and Yuda,M. (2012b). Centromere plasmid: a new genetic tool for the study of *Plasmodium falciparum*. PLoS. One. *7*, e33326.

Iwanaga,S., Khan,S.M., Kaneko,I., Christodoulou,Z., Newbold,C., Yuda,M., Janse,C.J., and Waters,A.P. (2010). Functional identification of the *Plasmodium* centromere and generation of a *Plasmodium* artificial chromosome. Cell Host. Microbe *7*, 245-255.

Janse,C.J., Kroeze,H., van,W.A., Mededovic,S., Fonager,J., Franke-Fayard,B., Waters,A.P., and Khan,S.M. (2011). A genotype and phenotype database of genetically modified malaria-parasites. Trends Parasitol. *27*, 31-39.

Khan,S.M., Kroeze,H., Franke-Fayard,B., and Janse,C.J. (2013). Standardization in generating and reporting genetically modified rodent malaria parasites: the RMgmDB database. Methods Mol. Biol. *923*, 139-150.

Lacroix,C., Giovannini,D., Combe,A., Bargieri,D.Y., Spath,S., Panchal,D., Tawk,L., Thiberge,S., Carvalho,T.G., Barale,J.C., Bhanot,P., and Menard,R. (2011). FLP/FRT-mediated conditional mutagenesis in pre-erythrocytic stages of *Plasmodium berghei*. Nat. Protoc. *6*, 1412-1428.

Laurentino,E.C., Taylor,S., Mair,G.R., Lasonder,E., Bartfai,R., Stunnenberg,H.G., Kroeze,H., Ramesar,J., Franke-Fayard,B., Khan,S.M., Janse,C.J., and Waters,A.P. (2011). Experimentally controlled downregulation of the histone chaperone FACT in *Plasmodium berghei* reveals that it is critical to male gamete fertility. Cell Microbiol. *13*, 1956-1974.

Lin,J.W., Annoura,T., Sajid,M., Chevalley-Maurel,S., Ramesar,J., Klop,O., Franke-Fayard,B.M., Janse,C.J., and Khan,S.M. (2011). A novel 'gene insertion/marker out' (GIMO) method for transgene expression and gene complementation in rodent malaria parasites. PLoS. One. *6*, e29289.

Pino,P., Sebastian,S., Kim,E.A., Bush,E., Brochet,M., Volkmann,K., Kozlowski,E., Llinas,M., Billker,O., and Soldati-Favre,D. (2012). A tetracycline-repressible transactivator system to study essential genes in malaria parasites. Cell Host. Microbe *12*, 824-834.

Sebastian,S., Brochet,M., Collins,M.O., Schwach,F., Jones,M.L., Goulding,D., Rayner,J.C., Choudhary,J.S., and Billker,O. (2012). A *Plasmodium* calcium-dependent protein kinase controls zygote development and transmission by translationally activating repressed mRNAs. Cell Host. Microbe *12*, 9-19.

Siden-Kiamos,I., Ganter,M., Kunze,A., Hliscs,M., Steinbuchel,M., Mendoza,J., Sinden,R.E., Louis,C., and Matuschewski,K. (2011). Stage-specific depletion of myosin A supports an essential role in motility of malarial ookinetes. Cell Microbiol. *13*, 1996-2006.

III. The Leiden Malaria Research Group SharePoint site: a rodent malaria parasite resource

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Overview

The Leiden SharePoint site/environment is a resource with relevance for researchers working with rodent malaria parasites and is maintained by the Leiden Malaria Research Group (LMRG). The information on the site is freely available, however, it is password protected. A password can be obtained on request by filling an access/password request form on the LMRG website (Figure 1). As of March 2013 the site has more than 400 registered members (principally malaria researchers from all over the world).

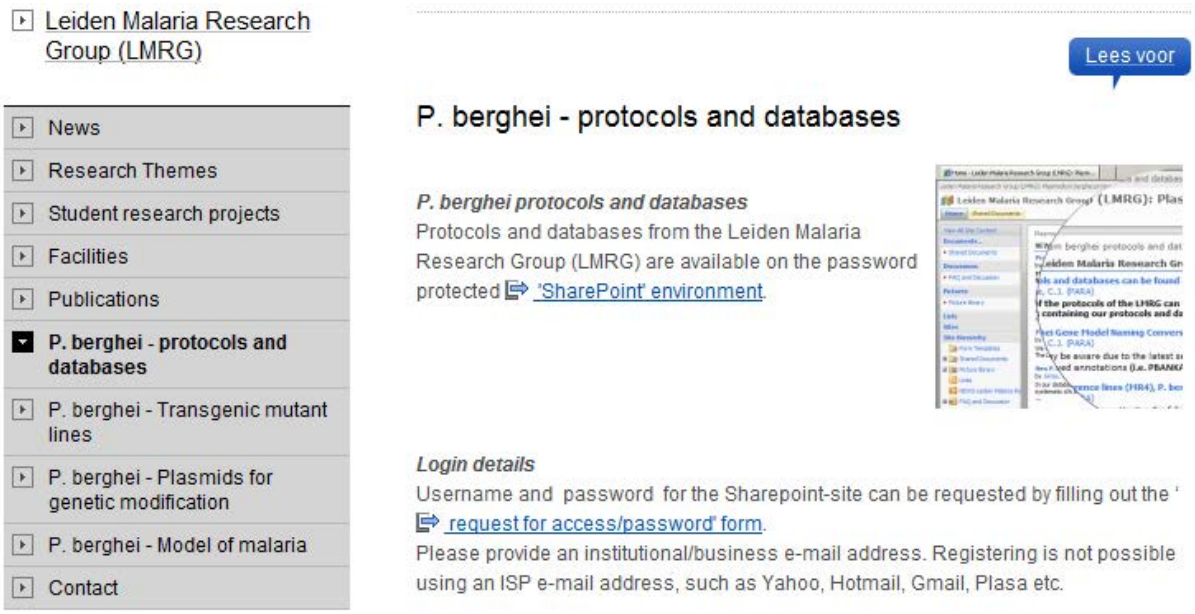


Figure 1: The LMRG website where it is possible to request access to the Leiden SharePoint site

What is in the LMRG SharePoint site?

The Leiden SharePoint site contains the following sections (Figure 2):
News: this contains all e-mails that have been sent to the registered SharePoint users describing new developments in transfection technology, news about the RMgm database of genetically modified rodent parasites etc. Some News examples are described below:

- GIMO transfection and negative selection: efficient generation of genetically modified malaria parasites
- *P. berghei* isolates, stabilates and laboratory lines: genotypes (genetic diversity) and growth and virulence phenotypes
- NOTIFICATION: incorrect integration of a LMRG positive/negative gene removal construct, pL0037, in the *P. berghei* genome

FAQ and Discussion: A number of answers to 'frequently asked questions' that have been received by the LMRG about working with rodent parasites. This includes questions about plasmids, transfection protocols, selection procedures to select for transfected parasites etc. Three examples of FAQ are shown below.

- Can the mice and rat diet influence *P. berghei* infections *in vivo* and compromise experimental design and interpretation?
- Which transgenic reporter *P. berghei* parasite line expressing luciferase is best used for whole body, real time, *in vivo* imaging of *P. berghei* schizont sequestration in live mice
- Do you have information on biosafety and animal health issues related to working with (transgenic) *P. berghei* parasites?

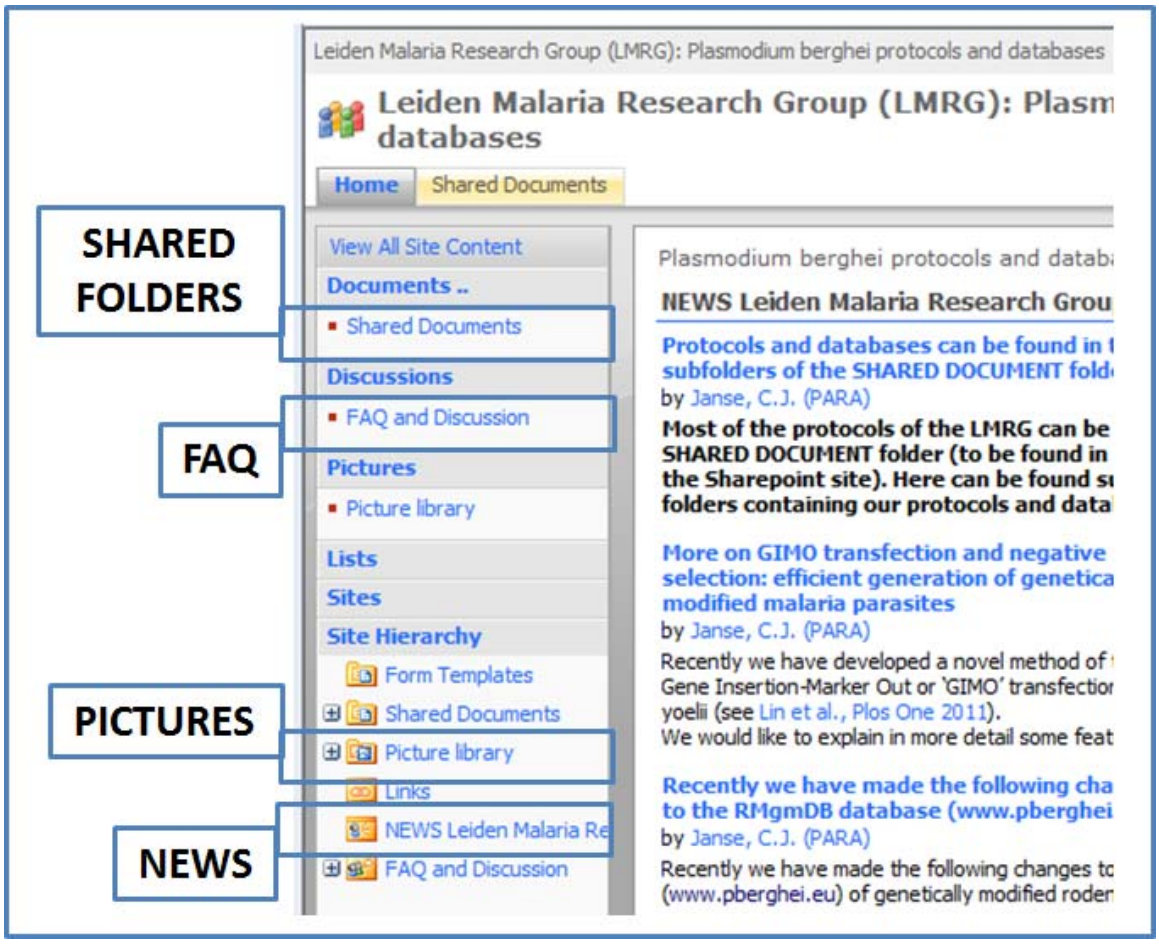


Figure 2: The different documents and resources can be accessed from different folders visible on the left hand column on the home page of the Leiden SharePoint site (see text for details of contents of the folders highlighted)
Picture Library: this contains images of plasmid maps from all (standard) plasmids available from the LMRG (see below)

Shared Folders: A number of folders providing laboratory protocols, datasets and background information on the malaria rodent models from LMRG (in WORD; Excel; PDF format). A few examples of information in different folders are given below (see also Figure 3).

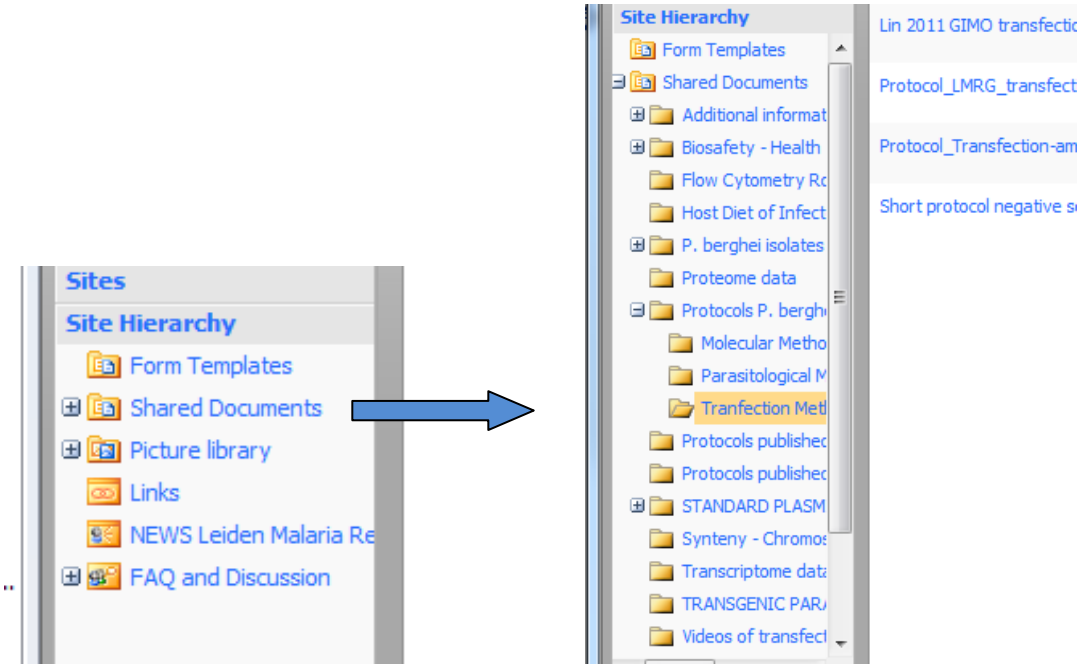
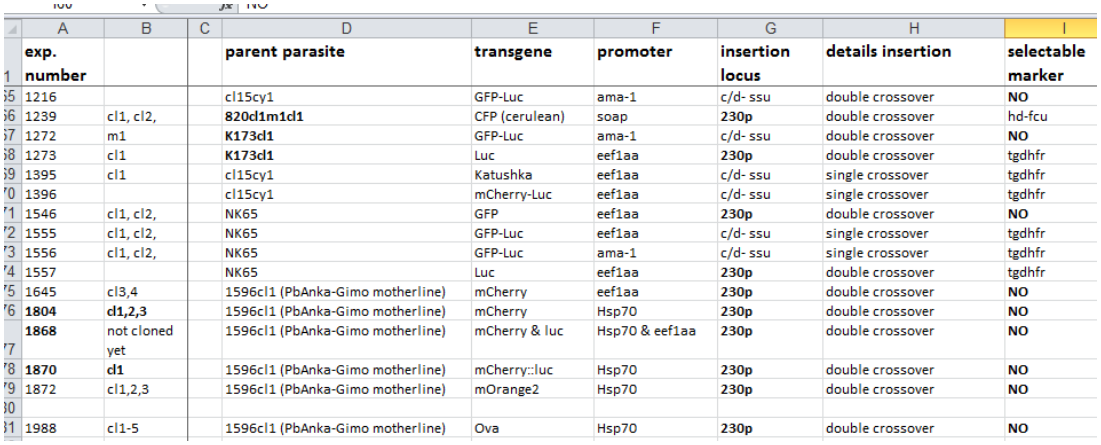


Figure 3. Selecting 'Shared Documents' folder on the Leiden SharePoint site reveals a number of subfolders (indicated by the blue arrow) where information and data on protocols, databases, rodent malaria parasite information etc. can be found

- *Protocols P. berghei*: Detailed *P. berghei* laboratory protocols (parasitological methods, molecular techniques, transfection technologies; videos of transfection protocols etc).
- *Biosafety - Health (transgenic) P. berghei in mice*: This includes general information about safety and health issues related to work with transgenic rodent malaria parasites. In addition, it provides health-testing reports of mice infected with reference LMRG transgenic lines (this also includes health-test reports obtained from other laboratories that have received the LMRG transgenic lines).
- *Host Diet of Infected Animals*: Information on host diet of laboratory animals infected with *P. berghei*. It is known that host diet can influence the course of *P. berghei* infections (and pathology) in mice.
- *P. berghei isolates and laboratory lines*: Information on the different field isolates/strains of *P. berghei* (strains available from the Edinburgh/Walliker collection; strains from Institute of Tropical Medicine, Antwerp etc). Information includes unpublished genotype data, data on gametocyte production, reticulocyte preference and unpublished sequence information on the following isolates K173, NK65, SP11

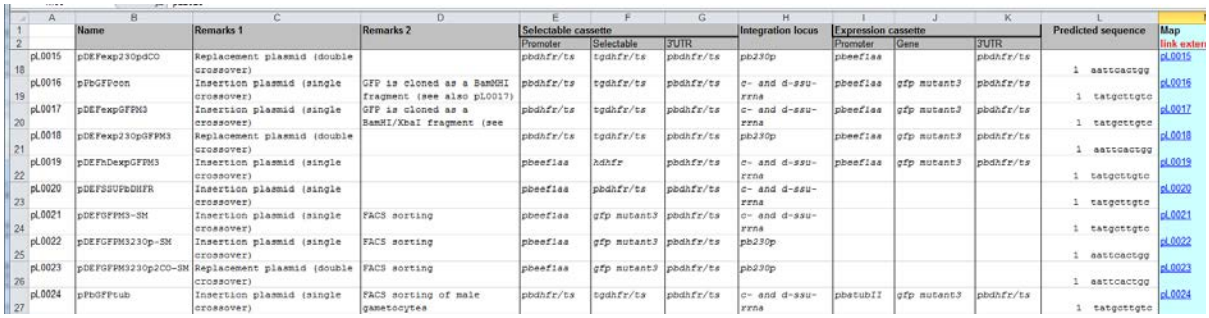
- *TRANSGENIC PARASITES available from LMRG*. Information of a large number of transgenic lines that have been generated in the LMRG and are available to the research community. These include for example transgenic lines that stably expressing different fluorescent and luminescent proteins under the control of different *Plasmodium* promoters (Figure 4). Many of these parasites do not contain a drug-selectable marker and are therefore suitable for additional genetic modification.



	A	B	C	D	E	F	G	H	I
1	**exp. number**			**parent parasite**	**transgene**	**promoter**	**insertion locus**	**details insertion**	**selectable marker**
55	1216			cl15cy1	GFP-Luc	ama-1	c/d- ssu	double crossover	**NO**
56	1239	cl1, cl2,		**820cl1m1cl1**	CFP (cerulean)	soap	**230p**	double crossover	hd-fcu
57	1272	m1		**K173cl1**	GFP-Luc	ama-1	c/d- ssu	double crossover	**NO**
58	1273	cl1		**K173cl1**	Luc	eef1aa	**230p**	double crossover	tgdhfr
59	1395	cl1		cl15cy1	Katushka	eef1aa	c/d- ssu	single crossover	tgdhfr
70	1396			cl15cy1	mCherry-Luc	eef1aa	c/d- ssu	single crossover	tgdhfr
71	1546	cl1, cl2,		NK65	GFP	eef1aa	**230p**	double crossover	**NO**
72	1555	cl1, cl2,		NK65	GFP-Luc	eef1aa	c/d- ssu	single crossover	tgdhfr
73	1556	cl1, cl2,		NK65	GFP-Luc	ama-1	c/d- ssu	single crossover	tgdhfr
74	1557			NK65	Luc	eef1aa	**230p**	double crossover	tgdhfr
75	1645	cl3,4		1596cl1 (PbAnka-Gimo motherline)	mCherry	eef1aa	**230p**	double crossover	**NO**
76	**1804**	**cl1,2,3**		1596cl1 (PbAnka-Gimo motherline)	mCherry	Hsp70	**230p**	double crossover	**NO**
77	**1868**	not cloned yet		1596cl1 (PbAnka-Gimo motherline)	mCherry & luc	Hsp70 & eef1aa	**230p**	double crossover	**NO**
78	**1870**	**cl1**		1596cl1 (PbAnka-Gimo motherline)	mCherry::luc	Hsp70	**230p**	double crossover	**NO**
79	1872	cl1,2,3		1596cl1 (PbAnka-Gimo motherline)	mOrange2	Hsp70	**230p**	double crossover	**NO**
80									
81	1988	cl1-5		1596cl1 (PbAnka-Gimo motherline)	Ova	Hsp70	**230p**	double crossover	**NO**

Figure 4. Examples of transgenic reporter lines available from LMRG; information of the line name and details of the reporter proteins, regulatory elements and selectable markers is also provided

- *STANDARD PLASMIDS for genetic modification*. A number of standard plasmids generated by the LMRG are available to the Research community (Figure 5). These include amongst others constructs to introduce different (fluorescent/bioluminescent) transgenes, constructs used for GIMO transfection in different *Plasmodium* species and strains etc.



	A	B	C	D	E	F	G	H	I	J	K	L	I
1		Name	Remarks 1	Remarks 2	Selectable cassette			Integration locus	Expression cassette			Predicted sequence	Map
2					Promoter	Selectable	3'UTR		Promoter	Gene	3'UTR		link exter
18	pL0015	pDEFexp230pdCO	Replacement plasmid (double crossover)		pbdhfr/ts	tgdhfr/ts	pbdhfr/ts	pb230p	pbeeflaa		pbdhfr/ts	1 aattcactgg	pL0015
19	pL0016	pPbGFPcon	Insertion plasmid (single crossover)	GFP is cloned as a BamHI fragment (see also pL0017)	pbdhfr/ts	tgdhfr/ts	pbdhfr/ts	c- and d-ssu-rrna	pbeeflaa	gfp mutant3	pbdhfr/ts	1 tatgcttgtc	pL0016
20	pL0017	pDEFexpGFPM3	Insertion plasmid (single crossover)	GFP is cloned as a BamHI/XbaI fragment (see	pbdhfr/ts	tgdhfr/ts	pbdhfr/ts	c- and d-ssu-rrna	pbeeflaa	gfp mutant3	pbdhfr/ts	1 tatgcttgtc	pL0017
21	pL0018	pDEFexp230pGFPM3	Replacement plasmid (double crossover)		pbdhfr/ts	tgdhfr/ts	pbdhfr/ts	pb230p	pbeeflaa	gfp mutant3	pbdhfr/ts	1 aattcactgg	pL0018
22	pL0019	pDEFhDexpGFPM3	Insertion plasmid (single crossover)		pbeeflaa	hdhfr	pbdhfr/ts	c- and d-ssu-rrna	pbeeflaa	gfp mutant3	pbdhfr/ts	1 tatgcttgtc	pL0019
23	pL0020	pDEFSSUPbDHFR	Insertion plasmid (single crossover)		pbeeflaa	pbdhfr/ts	pbdhfr/ts	c- and d-ssu-rrna				1 tatgcttgtc	pL0020
24	pL0021	pDEFGFPM3-SM	Insertion plasmid (single crossover)	FACS sorting	pbeeflaa	gfp mutant3	pbdhfr/ts	c- and d-ssu-rrna				1 tatgcttgtc	pL0021
25	pL0022	pDEFGFPM3230p-SM	Insertion plasmid (single crossover)	FACS sorting	pbeeflaa	gfp mutant3	pbdhfr/ts	pb230p				1 aattcactgg	pL0022
26	pL0023	pDEFGFPM3230p2CO-SM	Replacement plasmid (double crossover)	FACS sorting	pbeeflaa	gfp mutant3	pbdhfr/ts	pb230p				1 aattcactgg	pL0023
27	pL0024	pPbGFPtub	Insertion plasmid (single crossover)	FACS sorting of male gametocytes	pbdhfr/ts	tgdhfr/ts	pbdhfr/ts	c- and d-ssu-rrna	pbatubII	gfp mutant3	pbdhfr/ts	1 tatgcttgtc	pL0024

Figure 5. Examples of different DNA plasmid constructs available from LMRG and used in the generation of different rodent mutant parasites lines; information of the construct name, sequence, plasmid maps and details of the regulatory elements is also provided.

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