Middle Eastern Biology of Parasitism

University of Bern
Bern, Switzerland
July 23th - August 4th, 2018

Module II

Leishmania parasite

Diagnosis, molecular identification and drugs screening

Version 2

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Leishmaniasis MeBOP 2018 Agenda
Detection and characterization of parasites in patient samples, identification of vector blood meal preferences, and drug screening

Hour	**Sat July 28th**		**Sunday July 29th**	
12:00	DNA extraction from sand flies and mosquitoes			
14:00	Set up Leish /ITS1 / NGS-PCR		Set up microbiome / 16s / NGS - PCR	
	Set up SF/ COI / NGS-PCR		Set up mammalian CytB / NGS-PCR	
16:00				
17:00			Combine each 4 PCRs reactions in one tube	
			Purify PCR amplicons	
18:00			Addition of indices (O/N) PCR.	
19:00				
20:00				

Hour	**Monday July 30th**	
14:00	Amplicon purification	
15:00	Quality Control check	Passaging, counting cells ~1 hr
		Prep cells for DNA extraction
16:00	Send samples for NGS	
17:00	DNA extraction for ITS PCR and qPCR.	
18:00	Leishmania ITS PCR (classical)	
		Set up Drug assay for IC50 ~2hrs
19:00		With Amphotericin B and/or
		Paromomycin
20:00		
21:00		

Hour	**Tuesday July 31th**		**Wednesday 1st of August**	
14:00			***Swiss National Day***	
	Set up standard curve (qPCR/kDNA) 30 min			
15:00	Run kDNA qPCR ~ 1.5 hrs	Purification PCR products ~30min		
		ITS1 RFLP		
16:00		Set up Hae III digestion ITS1		
		Send for sequence analysis		
17:00				
	Run kDNA qPCR (HRM) O/N	Agarose gel electrophoresis		
18:00				
19:00				
20:00				
21:00				

Hour	**Tuesday July 31th**		**Friday August 3nd**	
14:00				
	Add Alamar Blue, measure	Introduction to NGS analysis	NGS analysis (our results)	Drugs screening (plate reading)
15:00	Fluorescence after 4 hrs			Fluorescence after 24 hrs
		Introduction to Galaxy		
16:00				
		DNA sequence analysis		
17:00				
	General discussion and loose ends	qPCR analysis and quantification		
18:00		qPCR/HRM analysis		
19:00				
20:00				
21:00				

Purification of total DNA from blood samples, biopsy materials, and insect tissue (spin-column protocol / DNeasy Blood & Tissue Kit).

Note: The original protocol is provided. For more detailed please refer to the *DNeasy Blood & Tissue Handbook* (www.qiagen.com/handbooks).

- **Blood samples** can be collected from suspected individuals as a finger-pricks or venous blood and spotted on 3MM Whatman paper.
- **Biopsy material** can be skin material collected on FTA membranes or bone marrow, liver and/or spleen biopsies kept frozen or in DNA lysis buffer.
- **Insects** can be stored whole in lysis buffer if DNA extraction will be performed within a few days, or kept in 70% alcohol for DNA extraction at a latter time.

Each group will prepare the following number of samples:

1 - 4 blood samples (spotted on filter paper).

2 - 4 biopsy materials (skin samples on filter paper).

3 - 4 sand flies that have blood (seen as red-dot in their abdomen).

4 – *L. tarentolae* promastigotes

5 - Samples that you brought to be tested for *Leishmania* infection.

Notes to be considered before starting the protocol:

- All centrifugation steps are carried out at room temperature in a microcentrifuge.
- Vortexing should be performed by pulse-vortexing for 5–10 s.
- **Blood samples** are digested in PBS (see step 1 below). **The ATL buffer is not used**.
- RNase A may be used to digest RNA during the procedure.
- Buffer AL may form a precipitate upon storage. If necessary, warm to 56°C until the precipitate is fully dissolved.
- Buffer AW1 and Buffer AW2 are supplied as concentrates. Before using for the first time, add the appropriate amount of ethanol (96–100%) as indicated on the bottle to obtain a working solution.
- Preheat a water-bath to 56°C.

Procedure:

1- Sample lysis:

- **Blood filter samples:** punch two discs of blood from the filter paper and transfer to a 1.5 ml tube. Add 180 µl PBS and 20 µl proteinase K. Proceed to step 2.
- **Skin biopsy materials:** punch two discs from the biopsy sample on the filter paper directly into the 1.5 ml tube. Add 180 ATL buffer and 20 µl proteinase K. Mix thoroughly by vortexing, and incubate at 56°C for 20 - 30 minutes or until the tissue is completely lysed. Go to step 2.
- **Insects (sand flies):** Add 180 ATL buffer and 20 µl proteinase K to each individual sand fly in a 1.5 ml tube. Sand fly tissue can be effectively disrupted before digestion using a rotor–stator homogenizer, or a plastic-rod that fits 1.5 ml tubes. Use one plastic-rod for each tube. Mix thoroughly by vortexing, and incubate at 56°C for 20-30 minutes or until the tissue is completely lysed. Proceed to step 2.
- **Promastigotes:** Take 0.5 ml of cultured promastigotes (~10^7 cells) and spin down by centrifugation (10 min, 1500 rpm). Suspend cell pellet in 200 µl PBS and add 20 µl proteinase K. Go to step 2.

Note: Lysis time varies depending on the type and size of tissue processed. Lysis is usually complete in 1–3 h, Longer incubation will not affect tissue adversely.

2. Add 200 µl Buffer AL to each sample, mix thoroughly by vortexing, and incubate at 56°C for 10 minutes**. Then add 200 µl ethanol (96–100%), and mix again thoroughly by vortexing.** It is essential that the sample, Buffer AL, and ethanol are mixed immediately and thoroughly by vortexing or pipetting to yield a homogeneous solution. A white precipitate may form on addition of Buffer AL and ethanol. This precipitate does not interfere with the DNeasy procedure.

4. Pipet the mixture from step 2 (including any precipitate) onto the DNeasy Mini spin column placed in a 2 ml collection tube (provided). Centrifuge at (8000 rpm) for 1 min. Discard flow-through and collection tube.

5. Place the DNeasy Mini spin column in a new 2 ml collection tube (provided), add 500 µl Buffer AW1, and centrifuge for 1 min at (8000 rpm). Discard flow-through and collection tube.

6. Place the DNeasy Mini spin column in a new 2 ml collection tube (provided), add 500 µl Buffer AW2, and centrifuge for 3 min at (14,000 rpm) to dry the DNeasy membrane. **Discard flow-through and collection tube.**

Note: It is important to dry the membrane of the DNeasy Mini spin column, since residual ethanol may interfere with subsequent reactions. This centrifugation step ensures that no residual ethanol will be carried over during the following elution. Following the centrifugation step, remove the DNeasy Mini spin column carefully so that the column does not come into contact with the flow-through, since this will result in carryover of ethanol.

7. Place the DNeasy Mini spin column in a clean 1.5 ml microcentrifuge tube, and pipet 100 µl Buffer AE directly onto the DNeasy membrane. Incubate at room temperature for 1 min, and then centrifuge for 1 min (8000 rpm) to elute.

Phenol extraction of DNA from blood, biopsy materials and insects

(will not be used in course)

- **Blood samples** can be collected from suspected individuals as a finger-pricks or venous blood and spotted on 3MM Whatman paper.

- **Biopsy material** can be skin material collected on FTA membranes, bone marrow, liver and/or spleen biopsies kept frozen or in DNA lysis buffer.

- **Insects** can be stored as whole in Lysis buffer if DNA extraction to be performed within few days, or kept in 70% alcohol for DNA extraction at a later time.

1. Add 200 µl of DNA lysis buffer to each tube, vortex or macerate the tissue in case of biopsy samples and insects.

   Lysis buffer: (50 mM NaCl, 10 mM EDTA, 50 mM Tris-HCl pH 7.4, 1% Triton X-100).

2. Add 20 µl of 10 mg/ml Proteinase K.

3. Incubate at 60^{o}C for 1-2 hour (until complete digestion of the tissue).

4. Add 0.2 ml Phenol solution (pH 8.0).

5. Vortex for 1 minute and spin at high speed (14,000 rpm) for 3 minutes.

6. Transfer the top aqueous layer into a new, labeled 1.5 ml microfuge tube.

7. Add NaCl to a final concentration of 0.2M (use 5 M NaCl, simply add 1 ml of 5 M NaCl for each 25 ml of transferred solution). Directly add about three volumes of cold absolute ethanol (Molecular biology grade).

8. Leave samples at -70^{o}C or at -20^{o}C for at least 30 minutes to precipitate the DNA. (At this stage samples can be kept for overnight).

9. Spin at high speed (14,000 rpm) for 10 minutes, 4^{o}C. A small pellet can be seen at this stage.

10. Remove alcohol from tubes and wash in 0.3 ml 70% alcohol.

11. Spin for 5 minutes at 4^{o}C, high speed (14,000 rpm).

12. Remove the alcohol and air-dry tubes or preferably dry in speed vacuum centrifuge if available.

13. Add 50 µl of sterilized double distilled water or 1X TE buffer (10 mM Tris, pH 8.0, 1 mM EDTA, pH 8.0).

14. Use 5µl of the prepared DNA for each PCR reaction.

This approach was adapted from the Nextera microbiome MiSeq DNA sequence protocol for use in detecting the *Leishmania* parasite in sand flies together with other vector associated elements such as blood meal analysis, sand fly species identification and microbiome analysis.

For this purpose, four different PCR reactions will be performed on each individual sand fly using special primers adapted for use in the Illumina MiSeq next generation sequencing (NGS) system. Each primer is composed of two parts: **First part:** Direct and reverse primers that are specifically designed to target the specific DNA to be amplified (examples: **a -** Leishmania ITS, **b -** mammalian cytochrome b gene, **c-** insects Cytochrome oxidase I gene (COI), and **d-** bacterial 16S rRNA gene). **Second part:** Universal 5' - tailed oligonucleotides (complementary region for R1 connected to the direct primer or R2 connected to reverse primer) known as Read 1 and Read 2. These sites will be used later for DNA sequencing from both sides.

In order to enable identification of individual sequences of different DNAs in pooled samples i.e., sand flies, dual barcode sequences are added to the ends of all DNA amplicons. These sequences, known as indices, allow for the identification of individual amplicons and their respective sequences for each sand fly. A unique index is used for each sand fly in the pooled sample.

So, as shown in the figure below for MiSeq analysis (taken from Microsynth: the Swiss DNA sequencing company), two PCRs need to be carried out: O**ne** - targeting a specific sequence (and could be many amplicons at the same time), and **Two** – addition of the dual bar code indices.

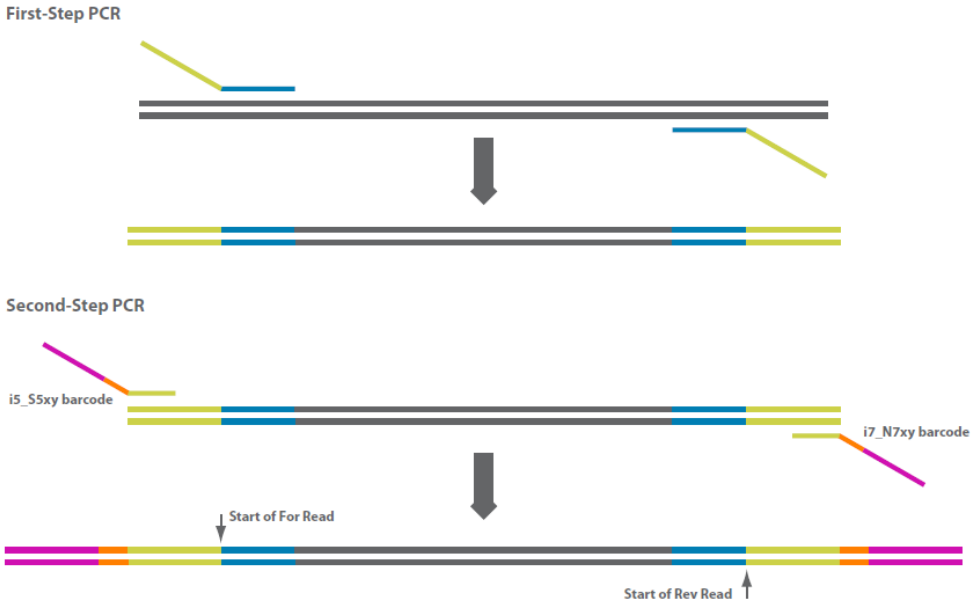


Figure 1. *Overview of the double indexing strategy used in the Illumina two-step protocol. During the Illumina sequencing step the amplified genomic sequence including the specific primers (grey and blue bars) as well as the forward and reverse barcodes (orange bar) are read out.*

Performing multiplex PCRs and preparation of MiSeq DNA amplicon library:

We are planning to analyze 90 sand fly samples, for each sand fly (SF) sample the following four PCRs will be performed:

1- *Leishmania* ITS-1 / NGS-PCR: for detection of infected SF samples (**Tm=56ºC**). Groups 1 and 2.
2- Mammalian cytochrome b / NGS-PCR: for identification of blood meal type (**Tm=56ºC**). Groups 3 and 4.
3- Insects' Cytochrome oxidase I (COI): for sand fly and mosquito identification (**Tm=53ºC**). Groups 6 and 6.
4- Bacterial 16s rRNA Microbiome; for bacterial identification (**Tm=60ºC**). Groups 7 and 8.

Every two groups will be responsible for one of the four PCR reactions. **DreamTaq Green PCR Master Mix (2X)** containing DNA polymerase, $MgCl_2$ and dNTPs will be used, and each group will prepare a reaction mixture sufficient for **50 PCR reactions** (45 samples and 3 negative controls).

PCR reaction mixture: The table below shows the quantities needed for one PCR reaction, supposing that *DreamTaq polymerase* is 2X concentrated and the final PCR reaction volume is 25µl.

Quantities needed for 1 PCR reaction

Material	**Quantity**
2X DreamTaq Mixture	12.5 µl
Forward primer / NGS	0.5 µl
Reverse primer / NGS	0.5 µl
*DNA****	*5 µl*
DDH2O	6.5 µl

*****Normally the master mix is prepared without the tested DNA, and later DNA is added to each individual sample tube.**

Calculations for 50 samples: The following table shows the needed quantities to carry out 50 PCR reactions (total volume of master mix = 1250 µl including total DNA samples volume).

Quantities needed for 50 PCR reactions

Material	**Quantity**
2X DreamTaq Ready Mix	625 µl
Forward primer	25 µl
Reverse primer	25 µl
dd H2O	325 µl

After preparing the master mix, aliquot **20 µl** to each PCR tube and add **5 µl** DNA from each sand fly sample to the tube. Negative control does not receive DNA, just ddH_2O.

Thermal cycler program:

- 5 min at 95ºC.
- 35 cycles: each composed of
    - 30 seconds at 95ºC.
    - 30 seconds ***at the PCR specific Tm, see above!!!!***
    - 1 min at 72ºC.
- A final elongation step at 72ºC for 10 min.

Representative samples (number to be discussed) will be analysed by Agarose gel electrophoresis for each PCR system.

PCR reaction pooling as a first step in MiSeq Library preparation:

Each group will be responsible for 16 individual sand flies, and will pool the PCR products from the four PCR reactions (for each fly make a pool containing the ITS, Cytob, COI and 16S rRNA amplicons). The mixed amplicons will be purified on AMPure XP beads according to the protocol provided below.

PCR products pooling and cleanup using AMPure XP beads (magnetic beads)

1- Make a pool of the four PCR products from each individual sand fly by taking 15 µl of each different PCR amplicon (except for the COI PCR product, only take 3 µl) and mixing in one tube. PCR pooling is carried out in 8 PCR tubes strips.

2- To each tube, containing the 48 µl of the pooled individual sand fly PCR products, add 30 µl of AMPure XP magnetic beads and mix well.

3- Keep the mixed components in the strips at room temperature for 5 minutes.

4- Transfer the strips to 96 well magnetic plate stand, and leave for another 5 minutes until the beads attach to tube side. (DNA is supposed to bind to the magnetic beads).

5- Remove solution by gentile pipetting making sure not to disturb the attached beads (if possible use the multichannel pipette).

6- Add 200 µl of freshly prepared 70% ethanol and leave for 1 minute while strips are not in the magnetic plate.

7- Return the strips to magnetic plate and let beads to attached to tube side (about 1 minute), and then remove the ethanol by pipetting.

8- Repeat washing with alcohol one more time, and at the end of the second wash leave strips containing the beads without any ethanol on the magnetic plates for about 5 minutes to dry.

9- Transfer strips to PCR tube strips holder, and then add 30 µl of double distilled water to elute bound DNA. Leave the beads in DDW for about 2-3 minutes.

10- Transfer strips to magnetic plate tube and then pipette the eluted DNA (20 µl) into fresh tubes.

Index addition by PCR: This PCR is used to attach the dual indices (i5 and i7) linked to Illumina sequencing adapters. For this PCR the indices names, indicated below, are written on the sample sheet that will be used (commonly they are named as: N7XX and S5XX). Index addition will be performed using ready dry taq polymerase (2x DreamTaq green Taq polymerase can also be used). The following is the composition of each PCR reaction: (reaction total volume= 15 µl).

Index PCR reaction mixture

Quantity	Material
Primer 1 - S5 XX index	**5 µl**
Primer 2 - N7 XX index	**5 µl**
DNA (quantity to be transferred directly to the tube at the end of magnetic bead purification).	**15 µl**
Total	**25 µl**

For easier mixing of the dual indices in each individual PCR tube arrange index primer 1 tubes (8 tubes of S5 XX) in a rack located in a vertical position, and index primer 2 tube (12 tubes of N7 XX) in another rack located in a horizontal position. Using multichannel pipette transfer 5 µl from each index to the specific PCR tubes (already containing the 15 µl pooled and purified PCR amplicons from the first stage PCR) as indicated in the sample sheet.

PCR on thermocycler using the following program:

- 5 min at 95ºC.
- **12 cycles: each composed of *(very important!!!!!)***
    - 30 seconds at 95ºC.
    - 30 seconds at 55ºC.
    - 30 seconds at 72ºC.
- A final elongation step at 72ºC for 5 min.

Representative samples (number to be discussed) will be analysed by Agarose gel electrophoresis in order to demonstrate dual index addition.

Final preparation of MiSeq pooled and barcoded sequencing library:

After addition of the indexes by the second PCR, all the reactions will be purified using the AMPure XP magnetic beads protocol, above, exactly as done after the first PCR. At the end of this purification step all the individual eluted PCRs will be pooled into one tube by mixing 10 µl from each eluted PCR product. At this stage the library is ready to be sent for next generation DNA sequencing (NGS) service company.

PCR assays for diagnosis and species Identification

The following PCR reactions will be carried out:

1. ***Leishmania***
    a. **ITS1 PCR assay.**
    b. **Cytochrome b PCR.**
2. **Blood meal analysis - mammalian cytochrome b PCR.**

- The exact PCR reaction protocol may change depending on the used *Taq DNA polymerase* used.
- Currently most of the companies sell *Taq enzyme* as a 2x concentrated ready mix, which includes: buffer, dNTPs, enzyme, and $MgCl_2$).
- It is recommended to suspend the extracted DNA (from blood samples, tissue, or sand fly) in 50 µl double distilled water, or TE buffer. For each PCR reaction 5µl of the extracted DNA will be used.
- Normally primers are diluted to a concentration of 20 pmoles/µl and 1µl (20 pmoles) of each primer are used in each PCR reaction.

PCR reaction mixture: The following are the quantities needed for one PCR reaction, supposing that *Taq polymerase* is 2x concentrated and the PCR volume to be prepared is 25 µl.

Material	**Quantity**
2X Taq Mixture	12.5 µl
Forward primer	1 µl
Reverse primer	1 µl
DNA	5 µl
DDH2O	5.5 µl

- **In most of the cases** more than one sample is tested, so enough master mix **without the DNA** is prepared first for all the samples, aliquot into tubes, and then DNA from each sample is added.

Example: For 9 samples prepare the reaction mixture as shown in the table below, plus 1 extra reaction to account for pipetting errors (Note: Positive and negative controls must be included in each reaction).

Material	Quantity For 10 reaction
2X Taq Ready Mix	125 µl
Forward primer	10 µl
Reverse primer	10 µl
dd H2O	55 µl

After preparing the master mix, aliquot **20 µl** to each PCR tube, then add **5 µl** DNA from each sample to the PCR tubes. Negative control does not receive DNA, just ddH2O.

Thermal cycler program:

- 5 min at 95°C.
- 35 cycles: each composed of
    - 30 seconds at 95°C.
    - 30 seconds at 56°C.
    - 1 min at 72°C.
- A final elongation step at 72 °C for 10 min.

Primers:

1) ***Leishmania* / ITS1 PCR assay.**
    a) **Forward - LITSR:** CTG GAT CAT TTT CCG ATG
    b) **Reverse - L5.8S:** TGA TAC CACTTA TCG CAC TT
2) ***Leishmania* cytochrome b PCR.**
    a) **Forward - LshCytoD:** TTG TAT GCA GAT AAT ATG TGG TGT GTG TTT AGC
    b) **Reverse - LshCytoR:** CCA TCT GAA CTC ATA AAA TAA TGT AAAC
3) **DNA amplification of mammalian cytochrome b gene.**
    a) **Forward - Cyto1:** CCA TCA AAC ATC TCA GCA TGA TGA AA
    b) **Reverse - Cyto2:** CCC CTC AGA ATG ATA TTT GTC CTC

References:

- El Tai, N.O, El Fari, M., Mauricio, I., Miles, M.I., Oskam, L.El Safi, S.H., Presber, W. and Schoenian, G. (2001). *Leishmania donovani*: Intraspecific Polymorphisms of Sudanese Isolates Revealed by PCR-based Analyses and DNA Sequencing. *Experimental Parasitology* **97**: 35–44

- Abbasi I, Cunio R, Warburg A (2008) Identification of Blood Meals Imbibed by Phlebotomine Sand Flies Using Cytochrome b PCR and Reverse Line Blotting. *Vector Borne Zoonotic Dis* 9(1): 79–86.

PCR Product Purification Protocol using Qiagene kit

- **Purpose:** Purification of PCR amplified DNA fragments for sequence analysis or for digestion with restriction enzymes. DNA fragments are purified to remove from reaction component ts(primers, nucleotides, polymerases, and salts) that may interfere with the next steps.

- **Important points before starting**

- Add ethanol (96–100%) to Buffer PE before use.
- All centrifugation steps are carried out at 13,000 rpm using a tabletop microcentrifuge at room temperature.

- **Procedure:**

1- Increase the volume of the PCR reaction up to 100 µl (add about 85 µl of DDW to each PCR tube to be purified).

2- Add 5 volumes of binding buffer PB to 1 volume of the PCR sample and mix. For example, add 500 µl of Buffer PB to 100 µl PCR sample.

3- Check that the color of the mixture is yellow. If the color of the mixture is orange or violet, add 10 µl of 3 M sodium acetate, pH 5.0, and mix. The color of the mixture will turn to yellow.

4- Place a QIAquick spin column in the 2 ml collection tube provided.

5- To bind DNA, apply the sample to the QIAquick column and centrifuge for 30–60s.

6- Discard flow-through. Place the QIAquick column back into the same tube.

7- To wash, add 0.75 ml Buffer PE to the QIAquick column and centrifuge for 30–60s.

8- Discard flow-through and place the QIAquick column back in the same tube. Centrifuge the column for an additional 1 min.

IMPORTANT: Residual ethanol from Buffer PE will not be completely removed unless the flow-through is discarded before this additional centrifugation.

9- Place QIAquick column in a clean 1.5 ml microcentrifuge tube.

10- To elute DNA, add **30 µl** Buffer EB (10 mM Tris·Cl, pH 8.5) or water (pH 7.0–8.5) to the center of the QIAquick membrane, let the column stand for 1 min, and then centrifuge for 1 min.

IMPORTANT: Ensure that the elution buffer is dispensed directly onto the QIAquick membrane for complete elution of bound DNA.

Elution efficiency is dependent on pH. The maximum elution efficiency is achieved between pH 7.0 and 8.5. When using water, make sure that the pH value is within this range, and store DNA at –20°C as DNA may degrade in the absence of a buffering agent. The purified DNA can also be eluted in TE buffer (10 mM Tris·Cl, 1 mM EDTA, pH 8.0), but the EDTA may inhibit subsequent enzymatic reactions.

Identification of *Leishmania* species is very important in regions when several species causing disease may be present, and knowledge of the species is necessary for appropriate public health measures and treatment. Ability to characterize the *Leishmania* species is also important when traveler's return from endemic regions where other species, different from local ones, exist, as these *Leishmania* may require different treatments regimens. Identification of *Leishmania* species in human and animal hosts, as well as in insect vectors, is important for epidemiological studies. Several molecular methods based on Polymerase chain reaction (PCR) have been developed for species identification. The most widely used methods rely on either restriction enzyme digestion (RFLP) or DNA sequence analysis, though other methods such as reverse line blot analysis or real-time PCR amplification followed by high resolution melt analysis can also be used.

DNA sequence analysis:

1- Purify ITS1-PCR and *Leishmania* cyto-PCR products that need to be identified. PCR cleaning can be carried out using the Qiagene PCR purification protocol. In the current example only purify the positive PCR clinical samples. There is no need to purify the *Leishmania* reference samples.

2- Samples are eluted in low volume (about 30 µl) of elution buffer or DDW. Do not dilute as high concentrations of purified DNA give better results.

3- For DNA sequencing of PCR products you need to use one of the two oligonucleotide primers in the amplification reaction (if only one strand is to be sequenced). Dilute your primers to 5 pmoles/µl, or according to the instructions of the DNA sequencing service provider.

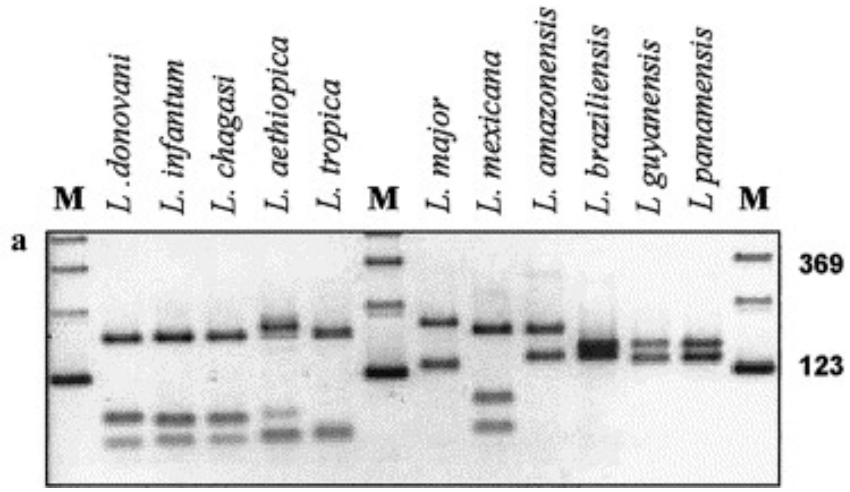
4- Once the sequence is received it is possible to carry out BLAST (*Basic Local Alignment Search Tool*) DNA sequence comparison on (*https://blast.ncbi.nlm.nih.gov/Blast.cgi*), and even generate phylogenic trees.

Note – the sequence file (*.abi) should be opened and the traces examined in order to determine the quality of the sequencing reaction and base calls.

ITS1-PCR restriction fragment length polymorphism (RFLP):

This method enables the identification of *Leishmania* species. It works best for Old World species: *L. donovani* complex - *L. donovani* and *L. infantum*, (synonym = *L. chagasi*), *L. aethiopica*, *L. tropica*, *L. major,* and *L. mexicana and L. amazonensis*. The ability to distinguish between species and subspecies in the *L. Viannia* subgenus (*L. braziliensis*, *L. guyanensis*, *and L. panamanensis,* etc.) is poor. Agarose gel electrophoresis works good enough for this analysis, but if it is possible to use capillary electrophoresis analysis it can provide better resolution and better distinguished band size.

Normally ITS1 digestion is carried out using *Hae*III, which gives distinct RFLP patterns for most *Leishmania* species, however additional restriction enzymes are occasionally used. A typical digestion of the ITS1–PCR amplicon from different species with *Hae*III is given in the figure, and the product sizes are given in the table



Digestion of amplified ITS1 regions of different *Leishmania* species with the restriction endonuclease *Hae*III. (*Shonian G. et. al. 2003*).

	L. donovani	***L. infantum***	***L. chagsi***	***L. aethiopica***	***L. tropica***	***L. major***	***L. turnica***	***L. mexicana***	***L. amazonensis***	***L. braziliensis***	***L. guyanensis***	***L. panamansis***
Band size (bp) obtained after				*200*	*185*	*203*	*203*					
	146	*184*	*184*	*57*	*57*	*132*	*57*	*186*	*186*	*156*	*156*	*156*
	75	*72*	*72*	*54*	*53*		*53*	*88*	*142*	*143*	*137*	*139*

digestion with *HaeIII* enzyme	*54*	*55*	*55*	*23*	*24*		*24*	*59*				

ITS1 - PCR digestion protocol using *HaeIII*:

1- Purify the ITS1 - PCR products according to the Qiagene PCR purification protocol.

2- It is highly recommended to include ITS-PCR amplified products from *Leishmania* reference samples for comparison purposes after analysis using agarose gel electrophoresis.

3- In a sterile 1.5 ml microfuge tube, prepare the restriction enzyme reaction mixture as indicated in table 2 below (the total volume is 20 µl/reaction), start with the needed amount of DDW to avoid enzyme denaturation. It is very important to keep all reagents on ice. Mix well or vortex the reaction mixture, and then centrifuge the tube for few seconds in order to spin down all the droplets.

Table 2. Reaction mixture for *Hae*III digestion.

Reaction component	**Volume**	**Notes**
10x reaction buffer	2 µl	1/10th of the reaction volume
DDW	7 µl	Or more depending on volume of DNA
*Hae*III enzyme	1 µl	Don't exceed more than 10% of the reaction volume, in order to avoid glycerol inhibition effect or no specific digestion.
Purified DNA	10 µl	Depends on DNA concentration

4- Incubate reaction tubes at 37°C for 1-2 hours in water bath.

5- Prepare a 2% agarose gel for analysis of the restriction digestion.

6- Make sure to include the digested PCR products from *Leishmania* reference samples and an appropriate molecular size marker (such as 100 bp ladder, or 50 bp ladder).

7- Load samples from each restriction enzyme digestion, one per lane.

8- Run the agarose gel at a voltage of 110-120 V, for about 30-50 minutes.

9- Examine the gel on UV light box and photograph.

Reference:

Schönian G, Nasereddin A, Dinse N, Schweynoch C, Schallig HDF, Presber W, Jaffe CL (2003). PCR diagnosis and characterization of Leishmania in local and imported clinical samples. Diagn. Microbiol. Infec. Dis. 47, 349-358.

Four steps are needed to perform this procedure:

1. PCR amplification of mammalian cytochrome b gene (see accompanying PCR protocol).

2. Immobilization of species specific mammalian probes to Biodyne C membrane.

3. DNA hybridization between the amplified PCR products and the membrane bound probes.

4. Detection of bound biotinylated PCR product

2. Immobilization of oligonucleotides to the membrane:

1. Cut Biodyne C membrane (15cm X 15 cm), this is enough for about 40-70 samples (depends on number of oligo-nucleotides to be used)
2. Wash membrane three times with 0.1 M HCL, 3min each wash time. The wash should be done on a shaker. Either side of the Biodyne C membrane will work for the oligonucleotide binding.
3. Wash three times with enough double distilled water (DDW), 2min each.
4. Incubate in a 5 - 10% EDAC solution for 30 minutes. This should also be done on a shaker set at a slow speed. Leave the membrane on Whatman 3MM filter paper to dry.
5. Dilute each Oligonucleotide to be used to a concentration of 5 pmol/µl in carbonate buffer (see below for recipe).
6. Place the treated membrane in the blotting apparatus and add ~ 180ul of the carbonate buffer to every other well. This will moisten the membrane and prevent the oligonucleotide samples from migrating out of their respective lanes.
7. Leave for about 5 minutes and then remove the remaining carbonate solution with a vacuum or a pipette.

8. Add carbonate buffer to the first lane and then the oligonucleotides to the rest of the lanes. In each lane add one specific oligo-nucleotide. In The last lane should also receive carbonate buffer, and to any other lanes that don't receive oligo-nucleotides.
9. Incubate for 30 minutes at room temperature. This will allow time for the oligonucleotides to bind to the membrane
10. Remove the oligonucleotide solution by vacuum or pipette, and allow membrane to air-dry for 10 minutes.
11. Wash with 0.1M NaOH for 5 minutes sharp**. If you wash for too long it will begin to remove the immobilized oligo-nucleotides.**
12. Wash three times with DDW; each time for about 5 min and dry the membrane on Whatman 3MM filter paper. The membrane can be stored at room temperature or at 4^{o}C for long-term storage.

3. DNA Hybridization and detection:

1. Cut strips of membrane; about 4mm width and perpendicular to the oligo-nucleotide lanes. As you cut the strips number the bottom of each strip using a sharp pencil.
2. Place strips in incubation trays. Each lane holds 3 ml total volume.
3. Add 2 ml of 2x SSC with 0.1% SDS. Incubate for 20 - 30 minutes at 45^{o}C with gentle shaking in the incubator. Longer incubation time will not affect the results.
4. While the membrane strips are incubating aliquot 0.5 ml of SSC/SDS solution into 1.5ml tubes, one tube per PCR reaction, and add the cytochrome b PCR products to the tubes. Place these tubes in a water-bath at 95^{o}C for 5 - 10 minutes to denature the biotinylated PCR products. Then immediately place tubes in an ice bath to prevent double stranded DNA renaturation.
5. Add the entire sample to the appropriate lane. Then incubate at 45^{o}C for 1 hour with a gentle shaking.
6. Remove entire solution from each lane using a pipette or vacuum. Then wash for 20 minutes with 2 ml of 0.7X SSC, 0.1% SDS at 45^{o}C with gentle shaking.
7. During the previous wash step prepare a 1:4000 dilution of Horseradish peroxidase - Streptavidin (HRP). The HRP should be diluted in 2X SSC, 0.1% SDS. Each lane needs about 2.5 ml.

8. Remove the wash solution from step 6 with vacuum or a pipette. Then add 2.5 ml of diluted HRP solution. Place on a shaker at room temperature for 30 minutes.
9. Remove HRP solution with vacuum or a pipette. Then wash 3 times with 2ml of 2x SSC, 0.1% SDS about 2 - 3 minutes for each wash. No need to use vacuum or pipette when removing washing solution here, it is possible to carefully drain the wash solution directly into the sink while retaining the membrane strips.
10. Add 2.5 ml of TMB solution (color detection of the hybridized PCR products) to the strips in the incubation tray and gentlely shake for about 10 minutes at room temperature. Blue color bands will start to appear in just a few minutes.

Important notes.

1. Always run negative control strips, and at least 2 known (positive) control strips.
2. The PCR should always be analyzed by agarose gel electrophoresis prior to RLB in order to make sure you have good PCR products.
3. During all steps prior to the RLB, DNA extraction and PCR, take all possible precautions to prevent DNA contamination. Keep in mind you are looking for human DNA products as well as well as other mammals.
4. Be sure to know the exact location of each oligonucleotide probe on the strip, exactly each which strips received which PCR product.

Materials and buffers:

- EDAC (Sigma E7750-25g): Store the EDAC powder frozen. Keep the solution refrigerated before use, and frozen during storage.
- Carbonate buffer 0.5M (Na_2CO_3 and $NaHCO_3$):
  - 4.2g $NaHCO_3$
  - 5.3g of Na_2CO_3

Dissolve in 80 ml DDW, and then adjust pH to 8.4, add DDW for a final volume of 100ml.

- 20x SSC

- 10% SDS.

-0.1M Na-Citrate.

- TMB hybridization detection solution:

    a. Prepare TMB (Sigma T8768) at 2mg/ml in ethanol. Store at 4°C.

    b. Dilute TMB in the following ratio

        - 19ml Na-Citrate (0.1M pH5.0), 1ml TMB, 2ul H_2O_2.

Alamar Blue assay for screening drugs on promastigotes

We will test for this experiment the effect of Paromomycin on viability of parasites

Needed:

a. Stock solution of 30 mM Paromomycin sulfate in water (sigma cat. No. P5057 or P9297)

b. Alamar Blue (BioRad cat No. BUF012A, Sigma, InVitrogen or other source)

c. Parasites

d. complete Medium

e. Microplate reader either fluorescent or absorbance (ELISA reader)

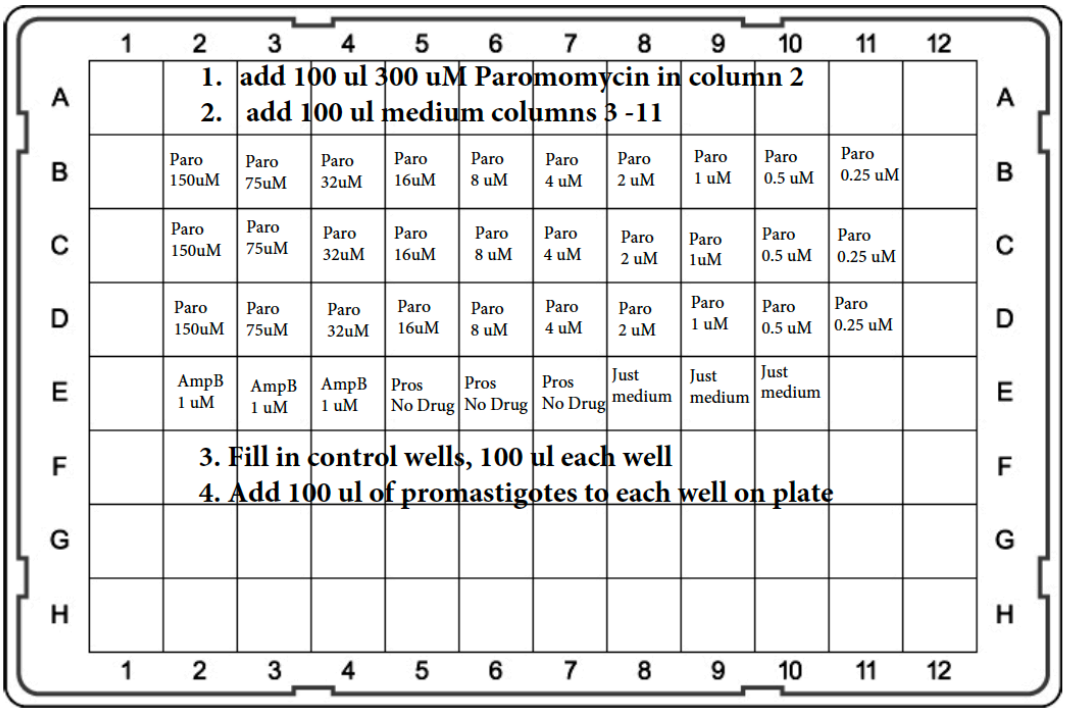
1. Count promastigotes
2. Adjust concentration of cells to ~2.5 X 10^6 / ml in complete medium
3. Aliquot 100 µl drug / well in triplicate using serial dilutions in medium starting at 300 µM to 1.0 µM
4. Aliquot 100 µl parasites / well in triplicate into a 96-well flat bottom plate (use appropriate plate for fluorescence or colorimetric detection depending on assay read out)
5. Control wells: Positive control - 1 µM Amphotericin B; Negative control – parasites no drug; Blank – Medium; optional control for interference – medium + test compound, no parasites
6. Close plate and incubate 48 hrs at 27ºC
7. Add 10% final concentration Alamar Blue solution (20 µl / well) and read fluorescence after 5 and 24 hrs (λex=530-560; λem=590). Note can also read on ELISA reader dual λ 570 nm / 620 nm reference.

Link to Alamar blue calculator: https://www.bio-rad-antibodies.com/colorimetric-calculator-fluorometric-alamarblue.html

Reference:

Shimony, O. and Jaffe, C.L. (2008). Rapid fluorescent assay for screening drugs on Leishmania amastigotes. J Microbiol Methods. ***75****, 196-200.*

Drug screening template



The main purpose of this test is quantitative the parasite load (number/ml) in blood samples or tissue biopsies of infected individuals. For this purpose a real-time kinetoplast DNA / qPCR assay (qRT-kDNA PCR) is used. Theoretically this assay can detect 1 parasite per ml of blood.

The protocol consists from three main stages:

A - Preparation of leishmanial standard curve by spiking of different parasite numbers in 1ml of human blood and spotting on filter paper.

B - DNA extraction from standard curve and unknown blood samples.

C - Running the quantitative real-time kinetoplast DNA PCR (qRT-kDNA PCR) and analyzing the results.

A. Setting up the *Leishmania* standard curve:

1 - Using an inverted microscope examine the *Leishmania tarentolae* (non-pathogenic) culture that will be provided in a sterile culture flask containing growth media. Make sure to see the main morphology of *Leishmania* organisms (promastigote stage), and examine carefully its motility and rosette formation behavior.

2 - Count the number of promastigotes by taking 20µl from the culture, mixing in 1:1 volume with the fixative solution. Load the fixed parasites in hemocytometer chamber, cover with special glass coverslips, and count the parasites in one of the corner squares (that consists of 16 smaller squares).

3 - Calculate the total number of parasites/ml by multiplying the number counted by 10^4 and the dilution factor (In this case 2).

4 - In 1.5 ml tubes, prepare the following concentrations of promastigotes in 1ml of sterile phosphate buffer saline (PBS), starting from 10^7, 10^6, 10^5, 10^4, 10^3, 10^2, 10, and no parasite.

5 - Using new 1.5 ml tubes, add 0.9ml of non-coagulated human blood in each of the 8 tubes.

6 - To each tube add 0.1 ml from the diluted promastigotes (from step 4), make sure to mix the promastigotes thoroughly before transfer to ensure even distribution of the parasites.

7 - Mix the promastigotes with the blood and take 50µl of the mixture to directly spot on Whatman 3MM paper. It is possible to make many (about 20) separated spots on the provided filter paper.

8 - Allow the blood spots containing the promastigote parasites to air dry for about 20 minutes. It is possible to use the spiked human blood directly in DNA extraction for preparing the standard curve, alternatively the filters can be well labeled, wrapped separately with aluminum foil and kept at -20^{o}C in plastic sacs containing silica gel until needed.

B - DNA extraction of parasites.

1 - Punch 2 discs from each of the eight filter paper leishmanial/blood standards Into separate tubes using one hole puncher (about 0.5 mm width).

2 - Similarly, punch 2 discs from any other samples to be tested. You will be provided with 4 filters containing leishmanial infected human blood with unknown parasite loads for testing.

3 - Add 180µl PBS and 20µl proteinase K, and then proceed with the DNA extraction using the Qiagene kit protocol for purification of total DNA from blood samples, as previously explained.

C - Running a quantitative real-time kinetoplast DNA PCR (qRT-kDNA PCR).

For this purpose a hot-start Absolute Blue qPCR kit (Thermo scientific, Surrey, UK) will be used. The detection of the PCR amplification products is based on detecting the fluorescent SYBR green dye once bound to the newly amplified double stranded DNA molecules. The reaction is run in a real time PCR thermocycler machine (such as: Rotor-Gene 6000, Qiagene, / or other equivalent machine).

1 - Count the number of the samples to be analyzed including the standard curve and negative no DNA controls. In general there are 8 points of different DNA concentrations used in creating the standard curve, two samples of no DNA control, plus the unknown samples to be tested: (in this case 4 samples per group). This results in 14 samples to be analyzed, so it is recommended to prepare a PCR mixture for 15 samples as indicated below. *Note that: the qPCR reaction has a total volume of 20µl, from which 2 µl will be the tested DNA sample*.

2 - qPCR reaction mixture: The following table shows the reaction mixture needed to prepare one 20µl reaction.

Material	**Quantity**
2X Absolute blue SYBR Green.	10µl
Forward primer (1µM)	1µl
Reverse primer (1µM)	1µl
Extracted DNA	2µl
DDH2O	6µl

3 - The following table shows the amounts needed to prepare master mix for 15 different samples:

Material	**Quantity**
2X Absolute blue SYBR Green.	150µl
Forward primer (10µM)	1.5µl
Reverse primer (10µM)	1.5µl
DDH2O	117 µl

Notes to be considered:

- *The total amount of the mix will be 300µl (which is: 15 samples X 20 µl; the total reaction volume).*
- *The forward and reverse primers are diluted in a final concentration of 10 µM.*
- *Each tube containing unknowns receives 2µl from the extracted DNA sample.*
- *No DNA samples receive 2 µl DDH2O.*
- *The total amount of DNA samples will be 30 µl (to be subtracted from the added amount of the final DDH2O).*

4 - Transfer 18 µl from the PCR master mixture to each individual qPCR tube.

5 - Add 2µl DNA from each samples to be tested to the appropriate tube, including samples from standard curve DNA.

6 - Run the qPCR machine according to the thermocycler program given below.

- qPCR thermal cycling program:

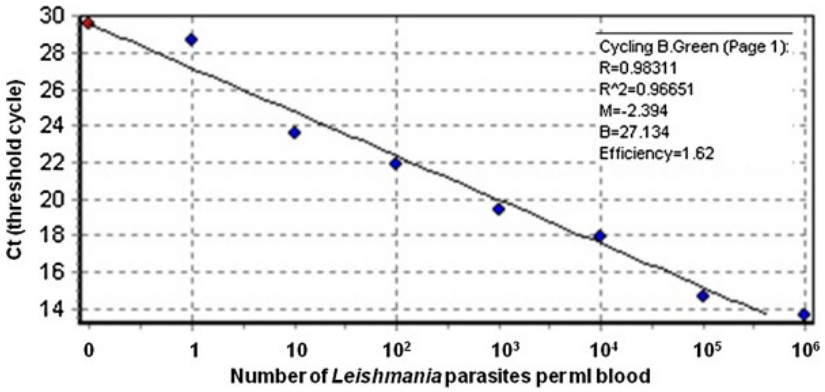
Step	**Temp.**	**Time**	**Number of cycles**
Enzyme activation	95 °C	15 min.	1 cycle
Denaturation	95 °C	10s	40 cycles
Annealing	58 °C	10s	
Extension	72 °C	20s	

Primers:

JW11: CCTATTTTACACCAACCCCCAGT

JW12: GGGTAGG GGCGTTCTGCGAAA

- Typical result showing an ideal standard curve:



A standard curve for qRT-kDNA PCR of *Leishmania donovani* promastigotes in blood. Human blood was mixed well, and dripped onto Whatman 3MM filter papers. On average, each drop (~50 µl) covered an area equivalent to 5 paper punch discs (r = 3 mm). Two discs were used for extracting DNA per reaction (~20 µl of blood). Standard curves were run with every batch of qRT-kDNA PCR and the number of parasites in tested samples was extrapolated from it.

Reference:

1- Nicolas, L., Milon, G., Prina, E., 2002, Rapid differentiation of Old World Leishmania species by LightCycler polymerase chain reaction and melting curve analysis. J Microbiol Methods 51, 295-299.

2- Abbasi, I., Aramin, S., Hailu, A., Shiferaw, W., Kassahun, A., Belay, S., Jaffe, C., Warburg, A., 2013. Evaluation of PCR procedures for detecting and quantifying Leishmania donovani DNA in large numbers of dried human blood samples from a visceral leishmaniasis focus in Northern Ethiopia. BMC Infect Dis 13, 153.