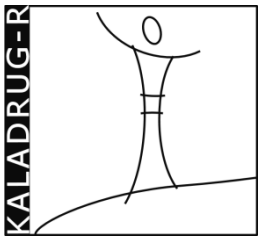
KALADRUG-R: Laboratory SOP #3 (version 147ITMxxx)



L. donovani specific PCR

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A. Aim

This protocol describes 2 PCRs that can be used for identification of *L. donovani* strains:

1. A PCR that specifically amplifies a fragment of the cysteine proteinase B type F (cpb-F) gene of *Leishmania donovani*. It does not amplify *L. infantum*, *L. major*, *L. tropica*, or *L. aethiopica*. The sensitivity is in general too low for typing clinical samples, but it is suited for parasite culture DNA. Cross-reaction has only been checked and validated against the aforementioned Old World species.

2. A PCR that specifically amplifies a fragment of the ribosomal internal transcribed spacer 1 (ITS1) of the *Leishmania donovani* complex, *i.e. L. donovani* and *L. infantum*, which cannot be discriminated using this method. It does not amplify *L. major*, *L. tropica*, or *L. aethiopica*. The analytical sensitivity is theoretically sufficient to analyze clinical samples, but this has not been validated, and the protocol here described is for DNA from promastigote culture. The method can be used to type DNA isolated from parasite cultures. Cross-reaction has only been checked and validated against the aforementioned Old World species.

Warning

These SOPs are provided as is, and the protocols presented have been extensively validated at ITMA. However, the species-specific nature of the here described PCRs is governed by minor DNA sequence differences between the species. As such, even slight modifications of the reaction conditions (both in composition and cycling parameters) or template amounts can result in **adverse** cross-reactivity, leading to erroneously typed parasites. It is therefore strongly recommended to validate the used PCRs in every setting on a panel of well-characterized isolates or strains to assess the specificity and sensitivity. The authors accept no liability for any misclassified parasites using these technologies.

B. PCR *cpb*-F

1. Make a list of parasite DNAs that have to be typed. Identify each DNA with a unique identifier.

2. Determine the DNA concentration of the parasite culture DNA samples spectrophotometrically by measuring the absorption at 260 nm.

3. Set up PCR mixes according to the following scheme:

Component	Amount Concentration
Water	Adjust to obtain final reaction volume of 30 µl
10x Coralload buffer, Qiagen Contains 1.5 mM $MgCl_2$	3.0 µl
Primer cpbF2.1 5′ GCGGCGTGATGACCAGC 3′	0.33 µM
Primer Do2.1 5′ CAATAACCAGCCATTCGTTTTTA 3′	0.33 µM
dGTP	200 µM
dATP	200 µM
dTTP	200 µM
dCTP	200 µM
HotStarTaq Plus DNA polymerase, Qiagen	1.5 units
DNA The exact DNA amount is not critical, but should be in the indicated range.	10-20 ng

Notes:

- A master mix must be prepared for all PCRs, including all components except DNA. After aliquoting the master mix, the respective DNAs are added in each tube.
- Include at least one positive control in each PCR run. This should be an *L. donovani* sample that is diluted as far as possible, but still amplifies in each PCR. **DO NOT** use a too concentrated sample, as this will still amplify even if the PCR reaction set-up is suboptimal. Use the same positive control DNA in each PCR, and for this reason use a DNA prep from which you have large quantities available.
- Include negative control samples to check for contamination in pre-PCR and template addition areas.
- In case another type of enzyme is used, the PCR must be validated for cross-reactivity to non-*L. donovani* parasites.

4. Run the following PCR cycle:

Cycles	Time	Temperature
1	5 min	95°C
40	30 sec 15 sec 15 sec	95°C 59°C 72°C
1	5 min	72°C

Note: As each PCR cycler and reagent tubes are different, the temperature profile of the mix might deviate from what has been tested in ITMA. The PCR should therefore be validated in each new setting prior to use.

5. Analyze 12 µl of each PCR in a 2.5 agarose gel, using an appropriate molecular weight marker as size reference. The *L. donovani* PCR product is 309 bp long. Coralload buffer already contains the necessary dyes.

6. If any of the negative control PCRs show a product of 309 bp, redo the PCR.

7. Identify samples showing a 309 bp product as *L. donovani.*

8. If the positive controls did not amplify a 309 bp product, redo the negative sample PCRs, as this might indicate sub-optimal PCR conditions.

9. Make an appropriate record of your experiment, results, and conclusion.

C. PCR ITS1

1. Make a list of parasite DNAs that have to be typed. Identify each DNA with a unique identifier.

2. Determine the DNA concentration of the parasite culture DNA samples spectrophotometrically by measuring the absorption at 260 nm.

3. Set up PCR mixes according to the following scheme:

Component	Amount Concentration
Water	Adjust to obtain final reaction volume of 25 µl
10x Coralload buffer, Qiagen Contains 1.5 mM $MgCl_2$	2.5 µl
Extra $MgCl_2$ In combination with the 1.5 mM included in the reaction buffer, this makes a total of 2.5 mM in the reaction.	1 mM
Acetylated BSA BSA: bovine serum albumin; to ensure DNase free preparations this is generally acetylated.	0.1 mg/ml
Primer rDNA-1F 5' AAACATATACAACTCGGGGAGA 3'	0.33 µM

Primer rDNA-1R 5′ TTACTGCAAATTTTGAGTACAAAAC 3′	0.33 µM
dGTP	200 µM
dATP	200 µM
dTTP	200 µM
dCTP	200 µM
dUTP	400 µM
HotStarTaq Plus DNA polymerase, Qiagen	0.5 units
DNA The exact DNA amount is not critical, but must be in the indicated range.	1-100 pg

Notes:

- A master mix must be prepared for all PCRs, including all components except DNA. After aliquoting the master mix, the respective DNAs are added in each tube.
- Include at least one positive control in each PCR run. This should be an *L. donovani* sample that is diluted as far as possible, but still amplifies in each PCR. **DO NOT** use a too concentrated sample, as this will still amplify even if the PCR reaction set-up is suboptimal. Use the same positive control DNA in each PCR, and for this reason use a DNA prep from which you have large quantities available.
- Include negative control samples to check for contamination in pre-PCR and template addition areas.
- In case another type of enzyme is used, the PCR must be validated for cross-reactivity to non-*L. donovani* parasites.

4. Run the following PCR cycle:

Cycles	Time	Temperature
1	5 min	95°C
40	30 sec 40 sec 40 sec	95°C 55°C 72°C
1	5 min	72°C

Note: As each PCR cycler and reagent tubes are different, the temperature profile of the mix might deviate from what has been tested in ITMA. The PCR should therefore be validated in each new setting prior to use.

5. Analyze 12 µl of each PCR in a 2.5 agarose gel, using an appropriate molecular weight marker as size reference. The *L. donovani* PCR product is 93 bp long. Coralload buffer already contains the necessary dyes.

6. If any of the negative control PCRs show a product of 93 bp, redo the PCR.

7. Identify samples showing a 93 bp product as *L. donovani* complex (*L. donovani* or *L. infantum*).

8. If the positive controls did not amplify a 93 bp product, redo the negative sample PCRs, as this might indicate sub-optimal PCR conditions.

9. Make an appropriate record of your experiment, results, and conclusion.