Training course

Molecular Epidemiology

Leishmaniasis

MANUAL

MOLECULAR PROCEDURES

18 - 24 May 2009

Instituto Oswaldo Cruz

Rio de Janeiro

Brazil

INTRODUCTION

Leishmaniases, both visceral (VL) and tegumentary (CL), are severe diseases that affect millions of people in the world. The current total number of cases estimated by the WHO is at 12 million, with 60.000 deaths and 2 million new cases every year. In Latin-America they can have a huge impact on the economic development prospects of the affected communities, which are often the poorest ones. However, leishmaniases remain virtually unknown by most of the general public and receive little attention from politicians and industry, and are thus considered “neglected diseases” by the WHO.

New tools for understanding of the complex and changing epidemiological settings of these diseases in Latin-America are required for scientifically based control programmes. Training of researchers and health workers in the use of these tools is an essential strategy to strengthen health and research institutions in these countries. The Training Course on Molecular Epidemiology of Leishmaniases held at Instituto Oswaldo Cruz, Rio de Janeiro, Brazil (18^{th}-24^{th} May, 2009) has been conceived with this aim.

This training course is integrated into the project “Control strategies for visceral leishmaniasis (VL) and mucocutaneous leishmaniasis (MCL) in South America: applications of molecular epidemiology (LeishEpiNetSA)”. This project, sponsored by the Commission of the European Communities (CEC), is coordinated by Prof Michael Miles of the London School of Hygiene and Tropical Medicine. The Consortium includes 6 European and 6 Latin-American Institutions.

The course has been designed for postgraduate Latin-American students and early stage researchers with the aims of a) introducing general epidemiological and population genetic methods associated with the leishmaniases and b) providing experience in a range of techniques available for the identification of *Leishmania*.

This manual presents standard methods used in our consortium as well as techniques recently developed by different members of the consortium for:

- extracting DNA from *Leishmania* cultures, vectors and clinical samples
- detection and identification of *Leishmania* species by different PCR-RFLP methods;
- *Leishmania* strain typing, such as multilocus sequence typing (MLST) and multilocus microsatellite typing (MLMT);
- in-silico sequence analyses and population genetic studies of parasites.

This course is aimed to enable the participants to implement the use of standardized methods in their endemic areas of study to facilitate comparisons of genotyping-based results.

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A. GENERAL LABORATORY PRACTISE & METHODS

1. Safety procedures

A. Chemicals

A number of chemicals used in the laboratory are hazardous. All manufacturers of hazardous materials are required by law to supply the user with pertinent information on any hazards associated with their chemicals. This information is supplied in the form of Material Safety Data Sheets or MSDS. This information contains the chemical name, CAS#, health hazard data, including first aid treatment, physical data, fire and explosion hazard data, reactivity data, spill or leak procedures, and any special precautions needed when handling this chemical. A file containing MSDS information on the hazardous substances used in the Molecular Biology course is kept in the lab.

In addition, MSDS information can be accessed on World Wide Web on the Biological Sciences Home Page. You are strongly urged to make use of this information prior to using a new chemical and certainly in the case of any accidental exposure or spill.

The instructor must be notified immediately in the case of an accident involving any potentially hazardous reagents.

The following chemicals are particularly noteworthy:

- Phenol: can cause severe burns
- Acrylamide: potential neurotoxin
- Ethidium bromide: carcinogen

These chemicals are not harmful if used properly: always wear gloves when using potentially hazardous chemicals and never mouth-pipette them. If you accidentally splash any of these chemicals on your skin, immediately rinse the area thoroughly with water and inform the instructor. Discard the waste in appropriate containers.

B. Ultraviolet light

Exposure to ultraviolet light can cause acute eye irritation. Since the retina cannot detect UV light, you can have serious eye damage and not realize it until 30 min to 24 hours after exposure. Therefore, always wear appropriate eye protection when using UV lamps.

C. Electricity

The voltages used for electrophoresis are sufficient to cause electrocution. Cover the buffer reservoirs during electrophoresis. Always turn off the power supply and unplug the leads before removing a gel.

D. General housekeeping rules

- All common areas should be kept free of clutter and dirty dishes, electrophoresis equipment, etc.

should be dealt with appropriately.

- Since you have only a limited amount of space, it is to your advantage to keep your own area clean.
- Since you will use common facilities, all solutions and everything stored in an incubator, refrigerator, etc. must be labelled. In order to limit confusion, each person should mark (i) his/her initials or other unique designation and (ii) date product was purchased/prepared. Unlabeled material found in the refrigerators, incubators, or freezers may be destroyed.
- Always mark vials, tubes, LB-plates etc. with your name/initials, the date, and relevant experimental data, e.g. strain numbers.

2. Preparation of solutions

A. Calculation of Molar, % and "X" Solutions

➲ A molar solution is one in which 1 litre of solution contains the number of grams equal to its molecular weight.

Example: to make up 100 ml of a 5 M NaCl solution (M.W. NaCl = 58.456 g/mol); dissolve 58.456 g x 5 moles x 0.1 liter = 29.29 g in 100 ml sol mole liter

➲ Percent solutions

Percentage (w/v) = weight (g) in 100 ml of solution

Percentage (v/v) = volume (ml) in 100 ml of solution.

Example: to make a 0.7% solution of agarose in TBE buffer, weight 0.7 of agarose and bring up volume to 100 ml with TBE buffer.

➲ "X" solutions

Many enzyme buffers are prepared as concentrated solutions, e.g. 5X or 10X =five or ten times the concentration of the working solution, and are then diluted such that the final concentration of the buffer in the reaction is 1X.

Example: to set up a restriction digestion in 25 µl, one would add 2.5 µl of a 10X buffer, the other reaction components, and water to a final volume of 25 µl.

B. Preparation of working solutions from concentrated stock solutions

Many buffers in molecular biology require the same components but often in varying concentrations. To avoid having to make every buffer from scratch, it is useful to prepare several concentrated stock solutions and dilute as needed.

Example: to make 100 ml of TE buffer (10 mM Tris, 1 mM EDTA), combine 1 ml of a 1 M Tris solution and 0.2 ml of 0.5 M EDTA and 98.8 ml sterile water.

The following is useful for calculating amounts of stock solution needed:

$$C_i \times V_i = C_f \times V_f$$

where C_i = initial concentration, or conc. of stock solution;

V_i = initial volume, or amount of stock solution needed;

C_f = final concentration, or conc. of desired solution;

V_f = final volume, or volume of desired solution.

C. Steps in solution preparation

1. Refer to the laboratory manual for any specific instructions on preparation of the particular solution and the bottle label for any specific precautions in handling the chemical.

2. Weigh out the desired amount of chemical(s). Use an analytical balance if the amount is less than 0.1 g.
3. Place chemical(s) into appropriate size beaker with a stir bar.
4. Add less than the required amount of water. Prepare all solutions with double distilled water.
5. When the chemical is dissolved, transfer to a graduated cylinder and add the required amount of distilled water to achieve the final volume. An exception is in preparing solutions containing agar or agarose. Weigh the agar or agarose directly into the final vessel.
6. If the solution needs to be at a specific pH, check the pH meter with fresh buffer solutions and follow instructions for using a pH meter.
7. Autoclave, if possible, at 121° C for 20 min. Some solutions cannot be autoclaved, for example, SDS. These should be filter sterilized through a 0.22 µm filter. Media for bacterial cultures must be autoclaved the same day it is prepared, preferably within an hour or two. Store at room temperature and check for contamination prior to use by holding the bottle at eye level and gently swirling it.
8. Solid media for bacterial plates can be prepared in advance, autoclaved, and stored in a bottle. When needed, the agar can be melted in a microwave, any additional components, e.g. antibiotics, can be added and the plates can then be poured.
9. Concentrated solutions, e.g. 1M Tris-HCl pH=8.0, 5M NaCl, can be used to make working stocks by adding autoclaved double-distilled water in a sterile vessel to the appropriate amount of the concentrated solution.

D. Glassware and plastic ware

Glass and plastic ware used for molecular biology must be scrupulously clean. Dirty test tubes, bacterial contamination and traces of detergent can inhibit reactions or degrade nucleic acid. Glassware should be rinsed with distilled water and autoclaved or baked at 150° C for 1 hour. For experiments with RNA, glassware and solutions are treated with diethyl-pyrocarbonate to inhibit RNases which can be resistant to autoclaving.

Plastic ware such as pipettes and culture tubes are often supplied sterile.

Tubes made of polypropylene are turbid and are resistant to many chemicals, like phenol and chloroform; polycarbonate or polystyrene tubes are clear and not resistant to many chemicals. Make sure that the tubes you are using are resistant to the chemicals used in your experiment. Micropipette tips and microfuge tubes should be autoclaved before use.

3. Disposal of Buffers and Chemicals

- **Uncontaminated**, **solidified agar** or agarose should be discarded in the trash, not in the sink, and the bottles rinsed well.
- **Media** that becomes **contaminated** should be promptly autoclaved before discarding it. Petri dishes and other biological waste should be discarded in biohazard containers which will be autoclaved prior to disposal.

- **Organic reagents**, e.g. phenol, should be used in a fume hood and all organic waste should be disposed of in a labelled container, not in the trash or the sink.
- **Ethidium bromide** is a mutagenic substance that should be treated before disposal and should be handled only with gloves. Ethidium bromide should be disposed of in a labelled container.

Dirty glassware should be rinsed, all traces of agar or other substance that will not come clean in a dishwasher should be removed, all labels should be removed (if possible), and the glassware should be placed in the dirty dish bin. **Bottle caps, stir bars and spatulas** should not be placed in the bins but should be washed with hot soapy water, rinsed well with hot water, and rinsed three times with distilled water.

4. Equipment

It is to everyone's advantage to keep the equipment in good working condition. As a rule of thumb don't use anything unless you have been instructed in the proper use. Rinse out all centrifuge rotors after use and in particular if anything spills. Please do not waste supplies - use only what you need.

Most of the experiments you will conduct in this laboratory will depend on your ability to accurately measure volumes of solutions using micropipettes. The accuracy of your pipetting can only be as accurate as your pipette and several steps should be taken to insure that your pipettes are accurate and are maintained in good working order.

1. DNA EXTRACTION

Protocol 1.1 **DNA extraction from cultured promastigotes using phenol-chloroform extraction**

Purpose:	to prepare *Leishmania* DNA from pelleted culture biomass and sand flies

A. INTRODUCTION:

The preparation of DNA includes DNA extraction, ethanol precipitation and DNA quantitation. The phenol-chloroform extraction is a thorough purification method that includes many steps of lysis, digestion, extraction and washing. Standard precipitation with ethanol is the standard method to recover nucleic acids from aqueous solutions. It is rapid and efficient for minute amounts of DNA and RNA. The most common method for quantitation of DNA is the spectrophotometric measurement of absorbance at 260 nm. It is rapid, simple and non-destructive, and can be used for pure samples that do not contain significant amounts of contaminants such as protein, phenol, agarose and other nucleic acids. The ratio of absorbance (OD_{260} : OD_{280}) is an indicator for the degree of contamination of the prepared DNA with protein and RNA/phenol.

B. MATERIALS:

Sample: culture biomass

Buffers and solutions:

⚠ CAUTION: Please see caution p.72
📖 RECIPE: Please see recipes p.68

- 📖 Lysis buffer
- ⚠ 📖 SDS (10% w/v)
- 📖 RNAse, 10 mg/ml
- 📖 Proteinase K, 20 mg/ml
- ⚠ 📖 Phenol:chloroform:isoamyl alcohol (25:24:1, v/v)
- ⚠ 📖 Chloroform:isoamyl alcohol (24:1, v/v)
- 📖 Ethanol 96 % and 70%
- Isopropanol
- 📖 Sodium acetate (3 M, pH 5.2)
- 📖 TE (pH 8.0)

Equipment and consumables:

Microfuge

Disposable pipettes

Waterbath or incubator

Refrigerator -20°C

1.5 ml and 2 ml microfuge tubes

Pipette tips (10, 100, 1000 µl)

C. METHOD:

1. Collect the promastigotes by centrifugation at 3000 rpm for 10 minutes.
2. Add 1- 2 ml of lysis buffer and resuspend the cells by gentle mixing. Do not vortex.
3. Transfer the suspension into a clean labelled 1.5 or 2 ml microfuge tube (use 2 tubes in case of 2 ml).
4. Add SDS to a final concentration of 0.5 % (20 fold-dilution i.e. 50 µl for a sample suspension of 1 ml) and shake well until the solution is viscous. Do not vortex.
5. Add RNase to a final concentration of 100 µg/ml (100 fold-dilution i.e. 10 µl for a sample suspension of 1 ml), incubate for 30 minutes at 37°C.
6. Add Proteinase K to a final concentration of 100 µg/ml (200 fold-dilution i.e. 5 µl for a sample suspension of 1 ml) and incubate at 60°C for at least 3 hours (better overnight).
7. Add an equal volume of phenol: chloroform: isoamyl alcohol mix to each tube. If necessary divide the suspension into two 1.5 ml microfuge tubes. Shake gently for at least 2-3 minutes. Do not vortex.
8. Centrifuge at 16000 g (max. speed) for 10 min and carefully transfer the aqueous phase to a clean labelled tube. Discard interface and organic phase. If the organic and aqueous phases are not well separated, centrifuge again for longer time.
9. Repeat steps 7 and 8 until the interphase disappears (normally 2 x phenol:chloroform: isoamyl alcohol is sufficient).
10. Add an equal volume of chloroform:isoamyl alcohol mixture to the aqueous phase, mix gently (do not vortex) and centrifuge as before. Carefully remove aqueous phase to a clean labelled tube and estimate its volume.
11. Add 1/10 volume of 3 M Na-acetate and 2-2½ volumes of ice-cold 96% ethanol (i.e. 200 µl sample + 20 µl 3 M Na-Acetate + 550 µl ice-cold 96% ethanol). Mix precipitate gently, do not vortex, and incubate at least 1hr (better overnight) at –20°C to allow the precipitation of DNA. Alternatively 0.6-1 volume of isopropanol can be used for DNA precipitation.
12. Centrifuge at max. speed for 30 min. and carefully remove the supernatant. Take care not to destroy the pellet of DNA, which may be invisible.
13. Wash the pellet by filling the tube with 70% ethanol. Do not shake or vortex, and re-centrifuge at max speed for 15 min in a microfuge.
14. Discard the supernatant and leave the open tube on the bench at room temperature until the last traces of fluid have evaporated. (Alternatively dry the DNA pellet by using a speed vacuum dryer for

5-10 minutes at 30°C).

15. Dissolve the DNA pellet in 100 µl (depending on the amount of DNA pellet) of either aqua bidest or TE buffer (pH 8.0) and store at 4°C until use. Highly concentrated DNA samples can be stored at 4°C for a long time.

D. DNA QUALITY & QUANTITY CHECK:

The **quality of DNA** can be visualised and the quantity estimated by running the DNA extracts on **agarose gels** (0.8-1.0 %) by using ethidium bromide in the gel or in the buffer.

1. Prepare an agarose gel as shown in protocol 2.1.
2. Load the gel as follows: 2 µl of extracted DNA + 2 µl loading buffer+ 10 µl aqua bidest or tank buffer (0.5xTBE). Use Lambda marker as molecular weight marker.
3. Run the gel at 100 V for 1 hour using a medium sized tray.
4. Photograph the gel under UV light (transilluminator).
5. Intact chromosomal DNA will appear as a distinct band at approx. 30kb. Compare the intensity of this band with standard bands for estimating the quantity of the DNA.

DNA concentration can be measured spectrophotometrically:

1. Spectrophotometers with deuterium lamps have to be turned on 30 minutes before the start of the measurement to allow the machine to warm up and stabilize.
2. Dilute the sample 1:20 (i.e. 5 µl sample + 95 µl distilled water).
3. Calibrate the spectrophotometer at OD_{260} and OD_{280} by blanking with distilled water.
4. Read the DNA sample at OD_{260} and OD_{280} using quartz cuvettes only. OD values should range between 0.1 and 1.0 to ensure an optimal measurement.

The concentration of a nucleic acid is determined by reading the optical density at 260 nm. OD_{260} = 1, corresponds to DNA concentration of 50 µg/ml. Thus calculate:

DNA concentration = OD_{260} x dilution factor x 50

The ratio between the optical densities at OD_{260} and OD_{280} is between 1.8 and 2 for pure DNA solutions. Values < 1.8 indicate contamination with proteins, values > 2 contamination with RNA and/or phenol.

REFERENCES:

Meredith SEO, Zijlstra EE, Schoone GJ, Kroon CCM, Van Eys GJ, Schaeffer KU, El Hassan AM, Lawyer PG. 1993. Development and application of the polymerase chain reaction for the detection and identification of *Leishmania* parasites in clinical material. Archives Institute Pasteur Tunis 70: 419-431

Protocol 1.2 **Preparation of DNA from clinical samples and sandflies using phenol-chloroform extraction**

Purpose: to prepare *Leishmania* DNA from clinical samples and sandflies for diagnostic PCR

A. INTRODUCTION:

The extraction of DNA from clinical samples and sandflies is challenging due to the different nature of biological material including fluids, solid and dry material; and due to the threat of contamination and carry-over between samples. The preparation of DNA from biological samples includes DNA extraction, ethanol precipitation and DNA purification. The phenol-chloroform extraction is a thorough purification method that includes many steps of lysis, digestion, extraction and washing. It is highly efficient in removing inhibiting factors, such as hemoglobin and is especially recommended for samples with relatively high amounts of blood. Precipitation with ethanol is the standard method to recover nucleic acids from aqueous solutions. It is rapid and efficient for minute amounts of DNA and RNA. Efficient extraction of DNA from clinical materials/sandflies and removal of inhibitors are crucial for the sensitivity of diagnostic PCR.

B. MATERIALS:

Sample: clinical specimen or sandflies

Buffers and solutions:

⚠ CAUTION: Please see caution p.72
📖 RECIPE: Please see recipes p.68

- 📖 Lysis buffer
- ⚠ 📖 Triton X-100
- 📖 Proteinase K, 20 mg/ml
- ⚠ 📖 Phenol:chloroform:isoamyl alcohol (25:24:1, v/v)
- ⚠ Chloroform:isoamyl alcohol (24:1, v/v)
- 📖 Ethanol 96 % and 70%
- Isopropanol
- 📖 Sodium acetate (3 M, pH 5.2)
- 📖 TE (pH 8.0)
- Purification kit

Equipment and consumables

Microfuge

Disposable pipettes

Waterbath or incubator

Paper puncher

Refrigerator -20°C

1.5 ml microfuge tubes

Pipette tips (10, 100, 1000 µl)

Razor blades or scalpels

C. METHOD:

1. Prepare sample for genomic DNA isolation:
   Bone marrow aspirate: Mix material approx. 1:1 with lysis buffer in a sterile microfuge tube.

   Biopsy / tissue materials: Dissect the tissue (minimum of 10-20 mg) and then either mince (chop) the tissue finely with a razor blade/scalpel or freeze the tissue in liquid nitrogen and grind it to a powder in a mortar pre-chilled with liquid nitrogen. The manipulated material is then mixed with 1-2 ml of lysis buffer. Take care to enhance the amount of Proteinase K added (2-5 times).

   Fresh blood samples: Collect 10-20 ml EDTA or ACD (Acid Citrate Dextrose, blood banks) blood (heparin is an inhibitor of PCR). Centrifuge for 10 minutes at 3000 rpm, aspirate the plasma and discard it. Carefully take the buffy coat and transfer it in a sterile labelled microfuge tube. Add one volume of lysis buffer.

   Filter paper samples: Using a sterile paper puncher (or 70% ethanol cleaned), punch out 2 discs from the blood drops on the filter paper and transfer them to a sterile labelled microfuge tube. Add 250 µl of lysis buffer. After each sample is obtained a clean sheet of paper (sprayed with or soaked in alcohol) should be punched 10-12 times in order to prevent DNA contamination from one sample to the next.

   Unstained / stained smears: Add 100 µl of lysis buffer to the tissue material adhering to the slide. Scrap off and mix using the tip and then aspirate the formed suspension from the surface of the slide and transfer it to a sterile labelled microfuge tube. Repeat the procedure once again using 150 µl of lysis buffer, making the total volume of lysis buffer 250 µl.

   Sandflies: Homogenise sandflies (thorax and whole abdomen of females) with sterile glass rods in 1.5 ml microfuge tubes containing 250 µl lysis buffer.
2. Add Triton X-100 to a final concentration of 1%.
3. Add Proteinase K to a final concentration of 100-200 µg/ml (or 400 µg/ml in case of tissue samples and sandflies) and incubate overnight at 60°C.
4. Add an equal volume of phenol:chloroform:isoamyl alcohol mix (25:24:1, v/v) to each tube. Shake gently for at least 2-3 minutes. Do not vortex.
5. Centrifuge at 16000 g (max. speed) for 10 min and carefully transfer the aqueous phase to a clean labelled tube. Discard interface and organic phase. If the organic and aqueous phases are not well separated, centrifuge again for longer time.
6. Repeat steps 4 and 5 until the interphase disappears (normally 2 x phenol:chloroform:isoamyl alcohol is

sufficient).

7. Add an equal volume of chloroform:isoamyl alcohol mixture (24:1, v/v) to the aqueous phase, mix gently (do not vortex) and centrifuge as before. Carefully remove aqueous phase to a clean labelled tube and estimate its volume.

8. Precipitate the DNA with ice-cold 96% ethanol by adding 1/10 volume of 3 M Na-acetate and 2-2½ volumes of 96% ethanol. Mix gently (do not vortex). Incubate 1hr (better overnight) at –20 °C to allow the precipitation of DNA. Alternatively 0.6-1 volume of isopropanol can be used for DNA precipitation.

9. Centrifuge at max. speed for 30 min. Remove the supernatant carefully. Take care not to disturb the pellet of DNA, which may be invisible.

10. Wash the pellet by filling the tube with cold 70% ethanol. Do not shake or vortex and re-centrifuge at max. speed for 15 minutes at 4 °C in a microfuge. Remove the supernatant carefully. Take care not to disturb the pellet of DNA, which may be invisible.

11. Discard the supernatant and leave open tube on the bench at room temperature until the last traces of fluid have evaporated. (Alternatively dry the DNA pellet by using a speed vacuum dryer for 5-10 minutes at 30 °C).

12. Dissolve the DNA pellet (which is often invisible) in 50-100 µl of either aqua bidest or TE buffer (pH 8.0). Diluted samples should be aliquoted and stored at -20°C until use.

13. To avoid inhibition during PCR, additional purification of the 100 µl DNA extract using commercially available kits like Nucleospin® Extract 2 in 1 from Macherey-Nagel-Germany (www.mn-net.com) or Qiagen PCR purification kit may be required. DNA samples will be eluted by using 30 µl of kit buffer.

14. Quality and quantity check of obtained DNA can be done as described in protocol 1.1.

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Protocol 1.3 DNA extraction of biological samples treated with Guanidine-HCl

Purpose: to preserve and prepare DNA from biological samples for further PCR analysis

A. INTRODUCTION:

Occasionally, biological samples are collected in the field and accurate preservation is needed for optimal DNA extraction and PCR analysis. Guanidine is a chaotropic agent which denatures proteins and inhibits DNAases activity. Treatment of the samples with guanidine-HCl will thus allow long time preservation at room temperature if the cold chain can not be maintained. Its chaotropic properties homogenise the total DNA in a given sample, for this reason an aliquot of a sample-guanidine lysate can be considered representative of original sample. When kDNA minicircles are used as PCR targets, boiling of samples preserved in guanidine-HCl disrupts the kDNA network and homogenises the minicircles in the solution.

B. MATERIALS

Sample: Biological sample.

Buffers and solutions:

Guanidine solution: Guanidine-HCl 6M / EDTA 0.2M pH 8.0

Equipment and consumables:

1.5 ml and 2 ml microfuge tubes

Waterbath

Pipettes and tips (10, 100, 1000 µl)

C. METHOD:

1. Mix properly 1 volume of biological sample with 1 volume of Guanidine-HCl solution. The ratio sample:guanidine can be increased up to 1:6, the latest preferable for samples with high nucleic acid content (spleen, liver aspirates).
2. Boil the mix for 15 min
3. Store the mix at RT, 4 ºC or -20 ºC; in dark.
4. Prior to DNA extraction boil the mix, again, for 15 min.
5. For DNA extraction use 100 µl of the lysate and add 300 µl of lysis buffer. Proceed as in a common Phenol/chloroform DNA extraction. Note that Proteinase K and detergent (SDS or Triton X-100) treatment will be needed for non-fluidic biological samples (biopsies).

2. AGAROSE GEL ELECTROPHORESIS

Protocol 2.1 **Normal agarose gels**

Purpose: to pour, load and run an agarose gel

A. INTRODUCTION:

The aim of this gel electrophoresis is to separate fragments of nucleic acids, DNA and RNA, and to detect them by staining with ethidium bromide and visualizing under UV light. The separation process is facilitated by electric current and based on molecular weight and charge. The most commonly used media in horizontal gels is agarose.

B. MATERIALS:

Sample: different DNA or PCR samples & DNA size standards (Lambda marker, ladder)

Buffers and solutions:

⚠ CAUTION: Please see caution p.72
📖 RECIPE: Please see recipes p.68

- 📖 Agarose
- 📖 TBE (Tris-borate-EDTA) buffer (0.5 X and 1 X)
- ⚠ 📖 Loading dye
- ⚠ 📖 Ethidium bromide (10 mg/ml)

Equipment and consumables:

Microwave

Balance

Waterbath

Magnetic stirrer

Disposable pipettes

Electrophoretic chamber and power supply

Transilluminator

Photo camera

Stirring bars

500 ml glass bottles or Erlenmeyer flasks

Gloves

Tape

Pipette tips (10, 100, 1000 µl)

C. METHOD:

1. Seal the edges of a clean, dry glass plate with a tape to form a mould. Set the mould on a horizontal section of the bench.
2. Prepare a sufficient amount of electrophoresis buffer to fill electrophoresis tank (0.5x TBE) and to cast the gel (1xTBE).
3. Weigh the appropriate amount of agarose powder (for instance 1.2 g for 1.2% agarose gel) in a clean 500 ml glass bottle or Erlenmeyer flask with a stirring bar.
4. Add 100 ml of **room temperature** 1xTBE buffer. The buffer should occupy max. 40 % of the volume of the flask or bottle.
5. Screw the cap **loose** in order to guarantee the pressure balance or, in case of flask, loosely plug the neck of the flask with kimwips.
6. Heat up the slurry in microwave (in the beginning at high power until bubbles appear, reduce to medium power later to avoid over boiling). Boiling waterbath can be used.
7. Weigh the flask and solution before heating.
8. Wear an oven mitten and carefully swirl the bottle or flask from time to time to make sure that any un-melted grains of agarose sticking on the walls enter the solution. Check for the **complete** dissolving of the agarose, until transparent solution is achieved.
9. Weigh the flask and solution again and check that the volume of the solution has not been decreased by evaporation during boiling; replenish with hot H_2O if necessary.
10. Use insulated gloves to transfer the flask/ bottle into water bath at 55 ^oC.
11. While the agarose gel is cooling, choose the appropriate comb for forming the sample slots in the gel. Position the comb 0.5-1 mm above the plate so that a complete well is formed when the agarose is added to the mould.
12. Pour the cooled agarose into the gel tray (mould) and wait till it solidifies.

    Hint: The gel should be between 3 mm- 5 mm thick. Check that no air bubbles are under or between the teeth of the comb. Air bubbles present in the molten gel can be removed easily by poking them with a tip or the corner of a Kimwipe.
13. Remove the tape and the two separating rulers from both ends. Mount the tray containing the gel in the electrophoresis tank, add just enough electrophoresis buffer (0.5x TBE) to cover the gel to a depth of 1 mm, and carefully remove the comb.
14. Mix the samples with water and loading dye up to a volume of 18-20 µl. For example, 5 µl DNA sample + 10 µl water +3 µl loading dye. PCR products from clinical samples may require a higher loading volume.
15. Slowly load the sample mixture into the slots of the submerged gel using a micropipette.
16. Close the lid of the gel tank and attach the electrical flexes so that the DNA will migrate towards the

positive anode (red flex). Apply a voltage of 4-5 V/cm (100-120 V) (measured as the distance between the positive and negative electrodes). Run the gel until the blue dye has migrated an appropriate distance through the gel.

17. At the end of the run turn off the electric current, and remove the flexes and the lid from the tank. Wash the gel in Aqua bidest. Submerge the gel in ethidium bromide (0.5 µg/ml) for 15 minutes and wash it again in Aqua bidest. Alternatively, ethidium bromide (final concentration of 0.5 µg/ml) can be added directly to the gel, after the molten gel has cooled.

    Hint: The migration of the loading dye depends from the type of agarose and its concentration as well as from the running buffer used (TBE or TAE).

    Hint: The presence of ethidium bromide in the gel allows it to be examined by UV illumination at any stage during the electrophoresis. The gel tray may be removed and placed directly on the transilluminator. Alternatively, the gel may be examined using a hand held source of UV light. In either case, turn off the power supply before examining the gel. During the electrophoresis, the ethidium bromide migrates towards the cathode (in opposite direction to that of the DNA). This can lead to loss of significant amounts of ethidium bromide from the gel, making detection of small fragments difficult. In this case staining the gel by immersion in buffer containing ethidium bromide is recommended. Also, having a separate ethidium bromide tank reduces the risk of contamination.

18. Examine the gel and photograph under UV light.

REFERENCES:

Recommendations of suppliers

Protocol 2.2 MetaPhor agarose gels

Purpose: to pour, load and run 4% MetaPhor agarose gels

A. INTRODUCTION:

MetaPhor agarose is a high resolution agarose that challenges polyacrylamide. Using submarine gel electrophoresis, PCR products and small DNA fragments that differ in size by 2% can be resolved.

B. MATERIALS:

Samples: RFLP fragments
Amplified microsatellite markers (PCR-products)
DNA size standards (100 or 50 bp ladder, 10 bp ladder)

Buffers and solutions:

⚠ CAUTION: Please see caution at p.72
📖 RECIPE: Please see recipes at p.68

- 📖 MetaPhor agarose
- 📖 TBE (Tris-borate-EDTA) buffer (1 X, 0.5 X)
- ⚠ 📖 Loading dye
- ⚠ 📖 Ethidium bromide (10 mg/ml)

Equipment and consumables:

Microwave

Balance

Waterbath

Magnetic stirrer

Disposable pipettes

Electrophoretic chamber and power supply

Transilluminator

Photo camera

Stirring bars

500 ml glass bottles or Erlenmeyer flasks

Gloves

Tape

Pipette tips (10, 100, 1000 µl)

C. METHOD:

1. Seal the edges of a clean, dry glass plate with tape to form a mould. Set the mould on a horizontal section of the bench.
2. Prepare a sufficient amount of electrophoresis buffer (0.5x TBE) to fill electrophoresis tank and to cast the gel (1 x TBE). Pour 100 ml of cold 1x TBE buffer in a glass bottle or Erlenmeyer flask that is 2-4 times the volume of the solution and add a stir bar.
3. Weigh the appropriate amount of Metaphor agarose powder (for instance 4 g for100ml buffer) in a clean beaker separately.
4. Add the agarose slowly to the buffer while the solution is rapidly stirred (magnetic stirrer) to prevent the formation of clumps.
5. Soak the agarose in the buffer for 10 min before heating. This reduces the tendency of the agarose solution to foam during heating.
6. Weigh the flask and solution before heating.
7. Screw the cap loose in order to guarantee the pressure balance or, in case of flask, loosely plug the neck of the flask with kimwips.
8. Heat the flask for 1-2 min (depends on the volume, it is 2 min for 200 ml) at maximal power but avoid boiling. Stir shortly using a magnetic stirrer.
9. Heat for 4 min at medium power, stir shortly and heat again for 4 min at medium power. The agarose should boil only slightly and be completely dissolved.
10. Check (by weighing) that the volume of the solution has not been decreased by evaporation during boiling. Replenish with hot H_2O if necessary (normally 10 ml per 200 ml solution).
11. Cool down for 2-3 min in a water bath at 50 ^{o}C on a magnetic stirrer.

    Hint: the agarose is quite viscous, use higher speed with the stirrer but avoid the formation of bubbles. Repeat stirring for another 2-3 min without water bath until all schlieres disappear.
12. While the gel is cooling, choose the appropriate comb for forming the sample slots in the gel. Position the comb 0.5-1 mm above the plate so that a complete well is formed when the agarose is added to the mould.
13. Pour the cooled agarose into the room temperature gel tray (mould) and let the gel solidify for 10-15 min at room temperature followed by 15 min in a refrigerator.

    Hint: The gel should be between 3 mm- 5 mm thick. Check that no air bubbles are under or between the teeth of the comb. Air bubbles present in the molten gel can be removed easily by poking them with the corner of a Kimwipe.
14. Remove the tape and the two separating rulers from both ends. Mount the tray containing the gel in the electrophoresis tank, add just enough electrophoresis buffer to cover the gel to a depth of 1 mm, and carefully remove the comb.
15. Mix the samples of DNA with water and loading dye up to a volume of 18-20 µl. For example, 5 µl DNA

sample + 10 µl water +3 µl loading dye.

16. Slowly load the sample mixture into the slots of the submerged gel using a disposable micropipette.
17. Close the lid of the gel tank and attach the electrical flexes so that the DNA will migrate towards the positive anode (red flex). Apply a voltage of 5 V/cm (140-150 V) (measured as the distance between the positive and negative electrodes). Run the gel until the blue dye has migrated an appropriate distance through the gel.
18. At the end of the run, turn off the electric current, and remove the flexes and the lid from the tank. Wash the gel in Aqua bidest. Submerge the gel in ethidium bromide (0.5 µg/ml) for 15 minutes and wash it again in Aqua bidest. Alternatively, ethidium bromide (final concentration of 0.5 µg/ml) can be added directly to the gel, after the molten gel has cooled.

    Hint: The migration of the loading dye depends from the type of agarose and its concentration as well as from the running buffer used (TBE or TAE).

    Hint: The presence of ethidium bromide in the gel allows it to be examined by UV illumination at any stage during the electrophoresis. The gel tray may be removed and placed directly on the transilluminator. Alternatively, the gel may be examined using a hand held source of UV light. In either case, turn off the power supply before examining the gel. During the electrophoresis, the ethidium bromide migrates towards the cathode (in opposite direction to that of the DNA). This can lead to loss of significant amounts of ethidium bromide from the gel, making detection of small fragments difficult. In this case staining the gel by immersion in Buffer containing ethidium bromide is recommended. Also, having a separate ethidium bromide tank reduces contamination
19. Examine the gel and photograph under UV light.

REFERENCES:

Recommendations of suppliers

3. DIAGNOSTIC PCR

Protocol 3.1 Conventional SSU PCR

Purpose: direct detection of *Leishmania* genus in clinical samples

A. INTRODUCTION:

This PCR targets a region conserved among all *Leishmania* species, the *SSU rRNA* gene, and is used for the direct detection, without prior cultivation, of the *Leishmania* genus in different types of clinical specimens. This PCR is available in 2 different formats: (i) a direct PCR (1 PCR round) and (ii) a nested-PCR (2 PCR rounds). For the nested PCR, the first round is not specific for *Leishmania*, a positive result needs to be confirmed by the second PCR round which uses *Leishmania* specific primers. Humans and dogs may be infected by lower trypanosomatids which show up as positive in the first PCR round, but negative in the second PCR round. The nested PCR format can be attractive because it has a greater sensitivity than the direct PCR and is therefore often used for diagnosis of human and canine leishmaniasis from different biological samples. In this manual we will only discuss the direct PCR, further reference on the nested PCR methods can be found at the end of this section.

B. MATERIALS:

Samples:
- DNA extracted from clinical samples
- *L. turanica* DNA for positive and inhibition controls
- Negative control (water)
- Negative preparation control (extraction protocol without biological material)

Buffers and solutions:

📖 RECIPE: Please see recipes p.68

- 📖 dNTP mix (2.5 mM) containing all four dNTPs
- 📖 10x amplification buffer
- 📖 *Taq* polymerase
- 📖 sterile distilled water
- 📖 sterile light mineral oil

Primers:		
R223	5´TCCCATCGCAACCTCGGTT 3´	15 pmol/µl
R333	5´AAAGCGGGCGCGGTGCTG 3´	15 pmol/µl

Table 1: Primer list SSU PCR

Equipment and consumables:

Thermocycler

Electrophoretic equipment

Transilluminator and photo camera

Microfuge or vortexer

Pipettes

Pipette tips (10, 100, 1000 µl, plugged)

PCR tubes 0.2 ml

C. METHOD DIRECT PCR (1 round):

Step 1: Prepare the Master Mix (MM) as indicated in the table below. Vortex and centrifuge the MM shortly and dispense the MM in pre-chilled labelled PCR-tubes.

MM (with reagents Roche GMP quality)		final concentration
10x PCR buffer (incl. 15 mM $MgCl_2$)	2.5 µl	1x (incl. 1.5 mM $MgCl_2$)
dNTP mix (2.5 mM)	2 µl	200 µM
primer R223 (15 µM)	0.5 µl	7.5 pmol
primer R333 (15 µM)	0.25 µl	3.75 pmol
Taq (5U/µl)	0.25 µl	1.25 U
H_20	14.5 µl	--
Total volume	20 µl	--

Hint: Multiply the quantity shown by the number of samples you have. Always prepare the MM for one sample extra i.e. if you have 5 samples, prepare for 6 so you have MM in excess to meet pipetting errors.

Table 2: Mastermix direct PCR SSU

Step 2: Add 5 µl of template DNA. The total reaction volume will be 25 µl, vortex and centrifuge the mixture briefly. Overlay (if necessary) the reaction mixtures with 1 drop (50 µl) of sterile light mineral oil to prevent

evaporation during the repeated cycles of heating and cooling.

Step 3: Prepare **positive**, **negative** and **inhibition controls** as indicated in table 3. Inhibition controls are run along with each clinical DNA sample to check for PCR inhibition due to co-extracted inhibitors. Inhibition controls are prepared by adding purified *L. turanica* DNA (same amount as in positive controls) AND clinical sample DNA to the MM. Comparisons of band intensities of positive and inhibition control will indicate whether PCR is inhibited or not.

	sample	inhibition control	+ control (*L. turanica*)	- control (DNA prep.)	- control (H_20)
MM	20 µl	20 µl	20 µl	20 µl	20 µl
DNA sample	5 µl	5 µl	--	--	--
L. turanica	--	2 µl	2 µl	--	--
blank DNA prep.	--	--	--	5 µl	--
distilled H_20	--	--	--	--	5 µl

Hint: The DNA concentration of *L. turanica* is 5 ng/µl. Each set of PCRs must always, always include positive (to monitor the efficiency of the PCR) and negative controls (to detect contamination).

Table 3: PCR controls to be included in each PCR run

Step 4: Run the following thermocycler programme:

cycle number	denaturation	annealing	Extension
1	5 min 94°C	--	--
35	30 sec 94°C	30 sec 65°C	30 sec 72°C
1	--	--	6 min 72°C
infinite	4°C		

Table 4: Amplification scheme for direct SSU PCR

Step 5: Check you PCR product on agarose gels. Examine the gel by UV light and photograph the gel. Expected product size ± 350 bp

REFERENCES:

Van Eys GJ, Schoone GJ, Kroon NC, Ebeling SB (1992). Sequence analysis of small subunit ribosomal RNA genes and its use for detection and identification of *Leishmania* parasites. Mol. Biol. Parasitol. 51:133-142

Cascio A, Calattini S, Colomba C, et al. Polymerase chain reaction in the diagnosis and prognosis of Mediterranean visceral leishmaniasis in immunocompetent children. Pediatrics **2002**; 109:E27.

Cruz I, Chicharro C, Nieto J, Bailo B, Canavate C, Figueras MC, Alvar J (2006). Comparison of new diagnostic tools for management of pediatric Mediterranean visceral leishmaniasis. J. Clin. Microbiol. 44:2343-2347

Cruz I, Canavate C, Rubio JM, Morales MA, Chicharro C, Laguna F, Jimemez-Mejias M, Sirera G, Videla S, Alvar J (2006). A nested polymerase chain reaction (Ln-PCR) for diagnosing and monitoring *Leishmania infantum* infection in co-infected patients with human immunodeficiency virus. Trans. Roy. Soc. Trop. Med. Hyg. 96 (Supp.1):185-189

Protocol 3.2 ITS1 PCR-sequencing and PCR-RFLP

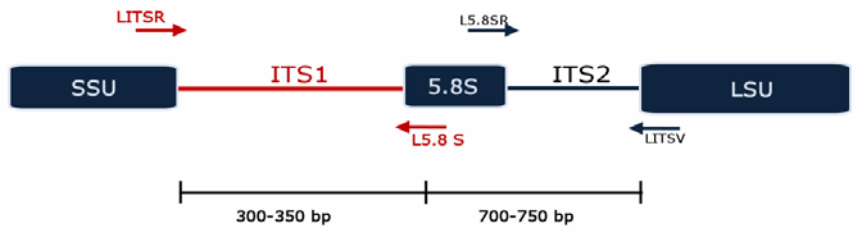
Purpose: to detect different *Leishmania* species using ITS1 PCR on DNA extracted from cultured promastigotes and clinical samples and to identify the *Leishmania* species by sequencing or digesting the ITS1 PCR product with endonuclease enzymes

A. INTRODUCTION:

High levels of inter and intra species variation have been observed in Old and New World *Leishmania* species in the DNA internal transcribed spacers (ITS1 and ITS2) present in the multi-copy ribosomal operon. The ITS1 PCR-RFLP (primer/product in red in figure below) is an example of a tool which exploits this feature and combines:

1. highly sensitive *Leishmania* diagnostic PCR suitable for use with different types of clinical samples including filter paper with bone marrow or lymph node aspirates, peripheral blood, skin scrapings, smashed sandflies etc.

subsequent species differentiation by sequencing or digestion of the ITS1 product with restriction endonucleases. The following species can be distinguished: *L. donovani*, *L. infantum*, *L. chagasi*, *L. aethiopica*, *L. tropica*, *L. major*, *L. mexicana*, *L. amazonensis*, *L. braziliensis*, *L. guyanensis*, *and L. panamanensis*.



region	primer pair	product size	primer sequence
whole ITS region	LITSR / LITSV	950-1130	5' CTGGATCATTTTCCGATG 3´ 5´ACACTCAGGTCGTAAAC 3´
ITS1	LITSR / L5.8S	300-350	5' CTGGATCATTTTCCGATG 3´ 5' TGATACCACTTATCGCACTT 3'
ITS2	L5.8SR / LITSV	700-750	5´ AAGTGCGATAAGTGGTA 3´ 5´ ACACTCAGGTCGTAAAC 3´

Table 8: Overview *Leishmania* ITS assays

Characterisation of *Leishmania* species in clinical infections is important, as different species may require distinct treatment regimens. Furthermore, such information is also valuable in epidemiologic studies where the distribution of *Leishmania* species in human and animal hosts, as well as in insect vectors, is a prerequisite of designing appropriate control measures.

B. MATERIALS:

Samples: DNA extracted from clinical samples or cultured promastigotes

L. turanica DNA for positive and inhibition controls

Negative control (water)

Negative preparation control (extraction protocol without biological material)

Buffers and solutions:

⚠ CAUTION: Please see caution at p.72

📖 RECIPE: Please see recipes at p.68

- 📖 dNTP mix (2.5 mM) containing all four dNTPs
- 📖 10x amplification buffer
- 📖 *Taq* polymerase
- ⚠ 📖 DMSO
- 📖 sterile distilled water
- 📖 sterile light mineral oil

restriction endonucleases *HaeIII*, *RsaI*, *HhaI* or *MnlI*

- 📖 10x restriction enzyme buffer

2% agarose of 2% Metaphor agarose gels

Primers:		
LITSR	5' CTGGATCATTTTCCGATG 3´	10 pmol/µl
L5.8S	5' TGATACCACTTATCGCACTT 3'	10 pmol/µl

Table 9: Primerlist ITS1 PCR

Equipment and consumables:

Thermocycler, PCR tubes 0.2 ml

Electrophoretic equipment

Transilluminator and photo camera

Microfuge

Vortex

Pipettes, Pipette tips (10, 100, 1000 µl, plugged)

DNA size standards

Waterbath or incubator

C. METHOD PCR:

Step 1: Prepare the Master Mix (MM) as indicated in the table below. Vortex and centrifuge the MM shortly and dispense the MM in pre-chilled labelled PCR-tubes.

	MM	final concentration
10x PCR buffer (incl. 15 mM $MgCl_2$)	5 µl	1x (incl. 1.5 mM $MgCl_2$)
dNTP mix (2.5 mM)	4 µl	200 µM
primer LITSR (10 µM)	2.5 µl	25 pmol
primer L5.8S (10 µM)	2.5 µl	25 pmol
DMSO	1.25 µl (*)	2.5%
Taq (5U/µl)	0.2 µl	1 unit
H_20	32.55 µl	--
Total volume	48 µl	--

Hint: Multiply the quantity shown by the number of samples you have. Always prepare the MM for one sample extra i.e. if you have 5 samples, prepare for 6 so you have MM in excess to meet pipetting errors. (*) optional, works better for clinical samples.

Table 10: Mastermix ITS1 PCR

Step 2: Add 2 µl of template DNA. The total reaction volume will be 50 µl, vortex and centrifuge the mixture briefly. Overlay (if necessary) the reaction mixtures with 1 drop (50 µl) of sterile light mineral oil to prevent evaporation during the repeated cycles of heating and cooling.

Step 3: Prepare **positive**, **negative** and **inhibition controls** analogous as in table 3; use 2 µl *L. turanica* DNA for the inhibition control. Inhibition controls are run along with each clinical DNA sample to check for PCR inhibition due to co-extracted inhibitors. Inhibition controls are prepared by adding purified *L. turanica* DNA (same amount as in positive controls) AND clinical sample DNA to the MM. Comparisons of band intensities of positive and inhibition control will indicate whether PCR is inhibited or not.

Step 4: Run the following thermocycler programme:

cycle number	denaturation	annealing	extension
1	2 min 95°C	--	--
33	20 sec 95°C	30 sec 53°C	1 min 72°C
1	--	--	6 min 72°C
infinite	4°C		

Table 11: Amplification scheme for ITS1 PCR

Step 5: Run 10 µl PCR product on 1% agarose gels or store the PCR product at -20°C.

Recommendation:

DNA extraction control (DEC)-true negativity test to check the integrity of DNA extracted from human clinical samples and rule out false-negative extractions. Run protocol 4.1 using one of the housekeeping genes such as β-actin or β-globin.

region	primer pair	product size	primer sequence	reference
Human β-globin	HβG-F/HβG-R	441	5' GAAGAGCCAAGGACAGGT AC 3' 5' CAACTTCATCCACGTTCACC 3'	Al-Jawabreh et al., 2004
Human β-actin	Aco1/Aco2	120	5´ACCTCATGAAGATCCTCACC 3´ 5´CCATCTCTTGCTCGAAGTCC 3´	Musso et al., 1996

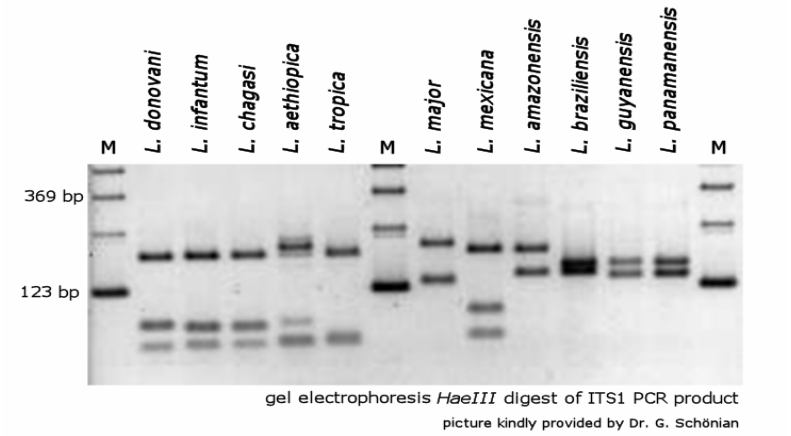
Table 12: Candidate primer pairs for DEC-true negativity test

D. METHOD: sequencing

Prior to sequencing the amplified fragments were purified by using the QIAquick PCR purification kit (QIAGEN) according to manufacturer's protocol.

Direct cycle sequencing was performed with the Taq DyeDeoxyTM Terminator Cycle Sequencing Kit in an automated sequencer A373 (Applied Biosystem) using the ITS1 primers and applying PCR conditions recommended by the manufacturer.

E. METHOD RFLP: *HaeIII* digestion of ITS1



gel electrophoresis *HaeIII* digest of ITS1 PCR product
picture kindly provided by Dr. G. Schönian

Normally, ITS1 digestion with one restriction enzyme results in distinct RFLP patterns for most *Leishmania* species. *L. donovani* complex strains and *Viannia* strains can be differentiated from other *Leishmania* species. Sequencing can be used for clear differentiation of all the species. Table 13 gives an overview of the restriction enzymes that are commonly used for *Leishmania* species identification based on ITS1 PCR product digestion. In this manual, we will only describe the *HaeIII*-digestion (indicated in red).

restriction enzyme ✂ fragments (bp)	L. donovani	L. infantum	L. chagasi	L. aethiopica	L. tropica	L. major	L. turanica	L. mexicana	L. amazonensis	L. braziliensis	L. guyanensis	L. panamanensis
HaeIII (used in this protocol)	164 75 54	184 72 55	184 72 55	200 57 54 23	185 57 53 24	203 132	203 57 53 24	186 88 59	186 142	156 143	156 137	156 139
HhaI	--	--	--	171 161 2	--	248 87	240 87 6	168 87 78	168 85 75	--	--	--
MnlI	--	--	--	--	276 43	173 109 43 10	--	284 49	--	259 40	253 40	255 40
RsaI	209 104	210 101	210 101	--	--	--	--	221 112	221 107	--	--	--

Table 13: Overview ITS RFLP profiles characteristic for the different species

Step 1: In a sterile 0.5 (1.5) ml microfuge tube, prepare the restriction mixture on ice, as indicated in the table below. Choose the recipe appropriate for the intensity of the PCR product obtained (as observed on gel). Keep all reagents on ice. Vortex the restriction mixture carefully and centrifuge it shortly to settle down all the droplets on the walls.

	MM digesting **10 µl** PCR product	MM digesting **15 µl** PCR product	MM digesting **20 µl** PCR product
10x buffer	1.5 µl	2 µl	2.5 µl
H_20	2.5 µl	2 µl	1.5 µl
HaeIII restriction enzyme (10 U/µl)	1 µl	1 µl	1 µl
Total volume	5 µl	5 µl	5 µl

Hint: (i) Multiply the quantity shown by the number of samples you have. Always prepare the MM for one sample extra i.e. if you have 5 samples, prepare for 6 so you have MM in excess to meet pipetting errors.
(ii) It is always good to add the buffer and water into the tube first. If you put the enzyme in straight on top of the buffer then it may become irreversibly denatured.
(iii) Do not use more enzyme than 10% of the final reaction volume. This is because the enzyme storage buffer contains antifreeze (glycerol) to allow it to survive at -20C. The glycerol will inhibit the digestion if present in sufficient quantities.
(iv) Depending on the enzyme used and from the manufacturer, it might be necessary to add bovine serum albumin (BSA).

Table 14: Set-up restriction digest ITS1 PCR

Step 2: Label sterile 0.5 ml microfuge tubes and dispense 5 µl of the reaction mixture in each tube. Add the adequate amount of ITS1 PCR product and mix. Centrifuge the mixture shortly to settle down all the droplets on the walls.

Step 3: Incubate at 37°C for 1-2 hours in waterbath or incubator. Meanwhile, prepare 2% Metaphor agarose (alternatively 1.5-2 % normal agarose) gel (protocol 2.1 or 2.2).

Step 4: After incubation, mix maximal 20 µl of the restriction mix with 3 µl of loading dye. This can be done by adding the loading buffer to the microfuge tubes.

Step 5: Slowly load the sample mixture into the slots of the submerged gel using a disposable micropipette, an automatic micropipette or a glass capillary tube. Into the first and the last slot load an appropriate molecular size marker (1kb -, 123 bp - or 100 bp- ladder).

Step 6: Run the agarose gel at a voltage of 4-5 V/cm (110-120 V) measured as the distance between the positive and negative electrodes (protocol 2.1).

Step 7: When the DNA samples or dyes have migrated a sufficient distance through the gel, turn off the electricity and remove the flexes and the lid from the tank. Examine the gel by UV light and photograph the gel.

Hint: Enzyme manufactures provide lots of information about restriction digestion. This is available both in the back of the catalogue and on the web:

www.neb.com; www.stratagene.com; www.promega.com; www.invitrogen.com; www.roche.com

REFERENCES:

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.

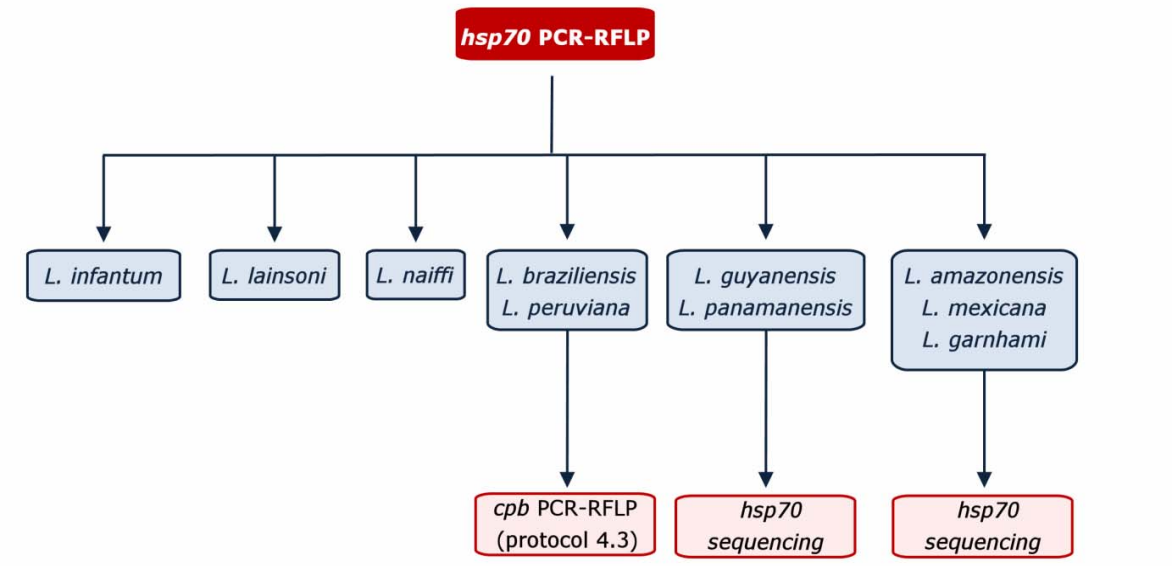
Kuhls, K., I. L. Mauricio, F. Pratlong, W. Presber and G. Schonian (2005). Analysis of ribosomal DNA internal transcribed spacer sequences of the Leishmania donovani complex. Microbes Infect 7(11-12): 1224-34.

Protocol 3.3 ***hsp70*** **PCR-RFLP**

Purpose: to detect different *Leishmania* species using *hsp70* PCR on DNA extracted from cultured promastigotes, clinical samples or sandflies and to identify the *Leishmania* species by digesting the *hsp70* PCR product with endonuclease enzymes

A. INTRODUCTION:

PCR-RFLP of the repeated *hsp70* genes is used for direct identification of neotropical *Leishmania* species present in clinical samples, such as skin scrapings collected with a tooth pick, or in insect tissues. In contrast to ITS1, *hsp70* encodes for a major antigen and thus allows probing for genetic variability of molecules possibly involved in immunopathology. The diagnostic sensitivity of the assay is around 95% and digestion of the *hsp70* PCR product with *HaeIII* discriminates 6 species/groups as indicated in the figure below. Further discrimination within each identified group, requires *hsp70* sequencing or *cpb* PCR – RFLP (see protocol 4.3).



The *hsp70* PCR-RFLP also identifies and distinguishes *T. cruzi* infections. The latter is useful to identify mixed infections of *Leishmania spp.* and *T. cruzi*, which can be quite frequent in Latin-America. The *hsp70* PCR-RFLP, in conjunction with the *cpb* PCR-RFLP (protocol 4.3), is now being introduced to support clinical and epidemiological monitoring of New World leishmaniasis.

B. MATERIALS:

Samples:

DNA extracted from clinical samples

DNA extracted from cultured promastigotes

DNA extracted from sandflies

L. turanica DNA for positive and inhibition controls

Negative preparation control (extraction protocol without biological material)

Buffers and solutions:

⚠ CAUTION: Please see caution at p.72
📖 RECIPE: Please see recipes at p.68

- 📖 dNTP mix (2.5 mM) containing all four dNTPs
- 📖 10x amplification buffer
- 📖 *Taq* polymerase
- ⚠ 📖 DMSO
- 📖 sterile distilled water
- 📖 sterile light mineral oil
- restriction endonuclease *HaeIII*
- 📖 10x restriction enzyme buffer
- 2% agarose or 2% Metaphor agarose gels

Primers:		
HSP70FOR	5' GACGGTGCCTGCCTACTTCAA 3'	10 pmol/µl
HSP70REV	5' CCGCCCATGCTCTGGTACATC 3'	10 pmol/µl

Table 15: Primerlist *hsp70* PCR

Equipment and consumables:

Thermocycler

Electrophoretic equipment

Transilluminator and photo camera

Microfuge

Vortex

Pipettes

Pipette tips (10, 100, 1000 µl, plugged)

PCR tubes 0.2 ml

DNA size standards

Waterbath or incubator

Microfuge tubes 1.5 ml

C. METHOD PCR:

Step 1: Prepare the Master Mix (MM) as indicated in the table below. Vortex and centrifuge the MM shortly and dispense the MM in pre-chilled labelled PCR-tubes.

	MM for DNA from clinical samples or sandflies	final concentration
10x PCR buffer (incl. 15 mM $MgCl_2$)	5 µl	1x (incl. 1.5 mM $MgCl_2$)
dNTP mix (2.5 mM)	4 µl	200 µM
primer HSP70FOR (10 µM)	2 µl	20 pmol
primer HSP70REV (10 µM)	2 µl	20 pmol
DMSO	2.5 µl	5.0%
Taq (5U/µl)	0.5 µl	2.5 unit
H_20	32 µl	--
Total volume	48 µl	--

Hint: Multiply the quantity shown by the number of samples you have. Always prepare the MM for one sample extra i.e. if you have 5 samples, prepare for 6 so you have MM in excess to meet pipetting errors.

Table 16: Mastermix *hsp70* PCR

Step 2: Add 2 µl of template DNA. The total reaction volume will be 50 µl, vortex and centrifuge the mixture briefly. Overlay (if necessary) the reaction mixtures with 1 drop (50 µl) of sterile light mineral oil to prevent evaporation during the repeated cycles of heating and cooling.

Step 3: Prepare **positive**, **negative** and **inhibition controls** analogous as in table 3; use 2 µl *L. turanica* DNA for the inhibition control.

Step 4: Run the following thermocycler programme:

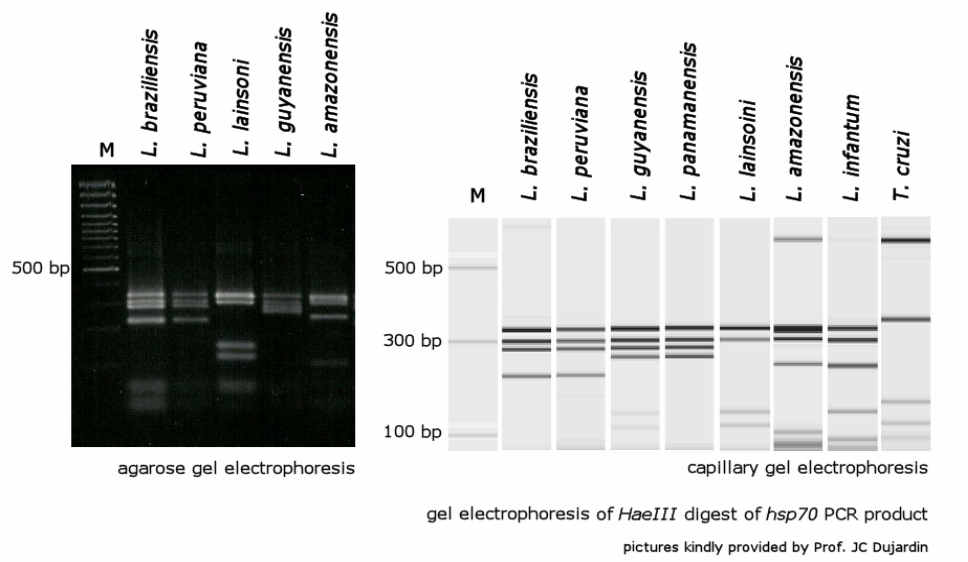
cycle number	denaturation	annealing	extension
1	5 min 94°C	--	--
35	30 sec 94°C	1 min 61°C	3 min 72°C
1	--	--	10 min 72°C
infinite	4°C		

Table 17: Amplification scheme for *hsp70* PCR

Step 5: Check you PCR product on 2% agarose, expected size is 1300 bp; or store at -20°C.

D. METHOD RFLP: *HaeIII* digest of *hsp70*

HaeIII digest of *hsp70* PCR products results in the following patterns:



gel electrophoresis of *HaeIII* digest of *hsp70* PCR product

pictures kindly provided by Prof. JC Dujardin

Step 1: In a sterile 0.5 (1.5) ml microfuge tube, prepare the restriction mixture on ice, as indicated in the table below. Choose the recipe appropriate for the intensity of the PCR product obtained (as observed on gel). Keep all reagents on ice. Vortex the restriction mixture carefully and centrifuge it shortly to settle down all the droplets on the walls.

	MM digesting **10 µl** PCR product	MM digesting **15 µl** PCR product	MM digesting **20 µl** PCR product
10x buffer	1.5 µl	2 µl	2.5 µl
H_20	2.5 µl	2 µl	1.5 µl
HaeIII restriction enzyme (10 U/µl)	1 µl	1 µl	1 µl
Total volume	5 µl	5 µl	5 µl

Hint: Multiply the quantity shown by the number of samples you have. Always prepare the MM for one sample extra i.e. if you have 5 samples, prepare for 6 so you have MM in excess to meet pipetting errors.

Table 18: Set-up restriction digest *hsp70* PCR

Step 2: Label sterile 0.5 ml microfuge tubes and dispense 5 µl of the reaction mixture in each tube. Add the adequate amount of *hsp70* PCR product and mix. Centrifuge the mixture shortly to settle down all the droplets on the walls.

Step 3: Incubate at 37°C for 1-2 hours in waterbath or incubator. Meanwhile, prepare 2% MetaPhor agarose (alternatively 1.5-2 % normal agarose) gel. (protocol 2.1 or 2.2).

Step 4: After incubation, mix maximal 20 µl of the restriction mix with 3 µl of loading dye. This can be done by adding the loading buffer to the microfuge tubes.

Step 5: Slowly load the sample mixture into the slots of the submerged gel using a disposable micropipette, an

automatic micropipette or a glass capillary tube. Into the first and the last slot load an appropriate molecular size marker (1kb -, 123 bp - or 100 bp- ladder).

Step 6: Run the agarose gel at a voltage of 4-5 V/cm (110-120 V) measured as the distance between the positive and negative electrodes (protocol 2.1).

Step 7: When the DNA samples or dyes have migrated a sufficient distance through the gel, turn off the electricity and remove the flexes and the lid from the tank. Examine the gel by UV light and photograph the gel.

Hint: Enzyme manufactures provide lots of information about restriction digestion. This is available both in the back of the catalogue and on the web:

www.neb.com; www.stratagene.com; www.promega.com; www.invitrogen.com; www.roche.com

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Protocol 3.4 kDNA minicircles (120 bp conserved region) PCR-RFLP

Purpose: to detect different *Leishmania* species using kDNA minicircles PCR on DNA extracted from cultured promastigotes and clinical samples and to identify the *Leishmania* species by digesting the 120 bp PCR product with endonuclease enzymes

A. INTRODUCTION:

In VL and ATL endemic areas where *L. (L.) chagasi*, *L. (L). amazonensis* and *L. (V.) braziliensis* are sympatric, it is important to have diagnostic tests which not only confirm the presence of parasite but also identify and distinguish the *Leishmania* species, thus allowing to address both clinical and epidemiological issues. The present method amplifies a c120 bp sequence of the conserved region of kDNA minicircles and further studies restriction fragment length polymorphism of the product obtained for each *Leishmania* species.

B. MATERIALS

Samples: DNA extracted from clinical samples or cultured promastigotes

L. turanica DNA for positive and inhibition controls

Negative control (water)

Negative preparation control (extraction protocol without biological material)

Buffers and solutions: Roche reagents, GMP quality

dNTP mix (10 mM)

10X amplification buffer (15 mM $MgCl_2$)

Taq polymerase (5 U/μl)

sterile distilled water

restriction endonuclease *HaeIII*

10x restriction enzyme buffer

2.5% agarose gel

Primers* (15 pmol/μl):

Forward: 5'- (G/C) (G/C) (C/G) CC (A/C) CTA T (A/T) TTA CAC CCA ACC CC-3´

Reverse: 5'- GGG GAG GGG CGT TCT GCG AA – 3'

* Note that these are degenerated primers, and different authors use minor variation of them (thus different names can be given to the primers).

Equipment and consumables:

Thermocycler

Electrophoretic equipment

Transilluminator and photo camera

Microfuge

Vortex

Pipettes

Pipette tips (10, 100, 1000 µl, plugged)

PCR tubes 0.2 ml

DNA size standards

Waterbath or incubator

Microfuge tubes 1.5 ml

C. METHOD PCR:

Step 1: Prepare the Master Mix (MM) as indicated in the table below. Vortex and centrifuge the MM shortly and dispense the MM in pre-chilled labelled PCR-tubes.

	MM	final concentration
10x PCR buffer (incl. 15 mM $MgCl_2$)	2.5 µl	1x (incl. 1.5 mM $MgCl_2$)
dNTP mix (10 mM)	0.5	200 µM
primer forward (15 µM)	0.5 µl	7.5 pmol
primer forward (15 µM)	0.5 µl	7.5 pmol
Taq (5U/µl)	0.25 µl	1.25 units
H_20	15.75 µl	--
Total volume	20 µl	--

Hint: Multiply the quantity shown by the number of samples you have. Always prepare the MM for one sample extra i.e. if you have 5 samples, prepare for 6 so you have MM in excess to meet pipetting errors. (*) optional, works better for clinical samples.

Step 2: Add 5 µl of template DNA. The total reaction volume will be 25 µl, vortex and centrifuge the mixture briefly. Overlay (if necessary) the reaction mixtures with 1 drop (50 µl) of sterile light mineral oil to prevent evaporation during the repeated cycles of heating and cooling.

Step 3: Prepare positive, negative and inhibition controls analogous as in table 3; use 2 µl *L. turanica* DNA for the inhibition control. Inhibition controls are run along with each clinical DNA sample to check for PCR inhibition due to co-extracted inhibitors. Inhibition controls are prepared by adding purified *L. turanica* DNA (same amount as in positive controls) AND clinical sample DNA to the MM. Comparisons of band intensities of positive and inhibition control will indicate whether PCR is inhibited or not.

Step 4: Run the following thermocycler programme:

cycle number	denaturation	annealing	extension
1	5 min 94°C	--	--
35	30 sec 94°C	30 sec 65°C	30 sec 72°C
1	--	--	5 min 72°C
infinite	4°C		

Step 5: Run 10 µl PCR product on 2.5% agarose gels or store the PCR product at -20°C.

D. METHOD RFLP: *HaeIII* digestion of kDNA *c*120 bp PRODUCT

HaeIII digestion of the 120 bp PCR product yields a distinctive pattern for *L. chagasi*, *L. amazonensis* and *L. braziliensis*.

Step 1: In a sterile 0.5 (or 1.5) ml microfuge tube, prepare the restriction mixture on ice, as indicated in the table below. Choose the recipe appropriate for the intensity of the PCR product obtained (as observed on gel). Keep all reagents on ice. Vortex the restriction mixture carefully and centrifuge it shortly to settle down all the droplets on the walls.

	MM digesting **10 µl** PCR product	MM digesting **15 µl** PCR product
10x buffer	1.5 µl	2 µl
H_20	2.5 µl	2 µl
HaeIII restriction enzyme (10 U/µl)	1 µl	1 µl
Total volume	5 µl	5 µl

Hint: (i) Multiply the quantity shown by the number of samples you have. Always prepare the MM for one sample extra i.e. if you have 5 samples, prepare for 6 so you have MM in excess to meet pipetting errors.

(ii) It is always good to add the buffer and water into the tube first. If you put the enzyme in straight on top of the buffer then it may become irreversibly denatured.

(iii) Do not use more enzyme than 10% of the final reaction volume. This is because the enzyme storage buffer contains antifreeze (glycerol) to allow it to survive at -20C. The glycerol will inhibit the digestion if present in sufficient quantities.

(iv) Depending on the enzyme used and from the manufacturer, it might be necessary to add

bovine serum albumin (BSA).

Step 2: Label sterile 0.5 ml microfuge tubes and dispense 5 µl of the reaction mixture in each tube. Add the adequate amount of kDNA PCR product and mix. Centrifuge the mixture shortly to settle down all the droplets on the walls.

Step 3: Incubate at 37^{o}C for 4 hours in waterbath or incubator. Meanwhile, prepare 2.5% agarose gel (protocol 2.2).

Step 4: After incubation, mix maximal 20 µl of the restriction mix with 3 µl of loading dye. This can be done by adding the loading buffer to the microfuge tubes.

Step 5: Slowly load the sample mixture into the slots of the submerged gel using a disposable micropipette, an automatic micropipette or a glass capillary tube. Into the first and the last slot load an appropriate molecular size marker (ladder including MWs below 100 bp).

Step 6: Run the agarose gel at a voltage of 4-5 V/cm (110-120 V) measured as the distance between the positive and negative electrodes (protocol 2.1).

Step 7: When the DNA samples or dyes have migrated a sufficient distance through the gel, turn off the electricity and remove the flexes and the lid from the tank. Examine the gel by UV light and photograph the gel.

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4. MULTILOCUS MICROSATELLITE TYPING (MLMT)

Protocol 4.1 Amplification and sizing of microsatellite markers

Purpose: to discriminate strains of the *Viannia complex* by using highly variable microsatellite markers

A. INTRODUCTION:

Microsatellites are tandem repeated stretches of short nucleotide motives of 1-6 bp ubiquitously distributed in eukaryotic genomes. They mutate at rates five to six orders of magnitude higher than the bulk of DNA. Microsatellite loci present high variability mainly due to allelic repeat length variation. After amplification with primers annealing specifically to their flanking regions, length variation of individual markers is estimated by using the fragment analysis tool of capillary sequencers, or screened on Metaphor or polyacrylamide (PAGE) gels. The results of these analyses are reproducible and exchangeable between laboratories.

Leishmania is relatively rich in microsatellites. Polymorphic repeats are however, not conserved between different species of *Leishmania.* Therefore, a new panel of 10-20 markers has to be developed for almost every species. Microsatellite markers are available for *L. donovani* complex, *L. tropica* and *L. major* (see references below) and for *Viannia* (unpublished). The markers for *Viannia* strains are 76-100 bp in size.

B. MATERIALS:

Samples: DNA extracted from cultured promastigotes, all at concentration of 10 ng/µl

10 bp ladder

Buffers and solutions:

RECIPE: Please see recipes at p.68

- dNTP mix (2.5 mM) containing all four dNTPs
- 10x amplification buffer
- *Taq* polymerase
- sterile distilled water
- 4% Metaphor agarose gel
- loading dye

Equipment and consumables:

Thermocycler, PCR tubes 0.2 ml

Electrophoretic equipment

Transilluminator and photo camera

Microfuge & vortex

Pipettes & pipette tips (10, 100, 1000 µl, plugged)

primerpair	primer sequences	annealing temperature	
CSg 46	5‘ AAACGTGCAAAGGCACATC 3‘ 5‘ TCTATTACCGCGCTCATGCT 3‘	54°C	10 µM
CSg 47 *	5‘ GTGTTCGTGAAACGTCGAAA 3‘ 5‘ AAAAGGCCGGTTTCAAATTC 3‘	56°C	10 µM
CSg 48 *	5‘ TTGACGTGTACACCGCTCTT 3‘ 5‘ TTCTGAGAAAGGCAACCGATA 3‘	56°C	10 µM
CSg 53 *	5‘ CATGTAGGCATGCGGTTGTA 3‘ 5‘ GCTCCTTTTCTCGTTTGAAC 3‘	54°C	10 µM
CSg 55 *	5‘ GCTTTGCTTGGACTGGAGAG 3‘ 5‘ GGAGGGAAAAGGAAGTCCAG 3‘	56°C	10 µM
CSg 59 *	5‘ CATTTGAGCTGCACGTGTCT 3‘ 5‘ AACGCAATGGTCGGTACTTC 3‘	56°C	10 µM
7GN	5‘ TCTTTCCGCTACGTGGTTG 3‘ 5‘ AACGCAATGGTCGGTACTTC 3‘	54°C	10 µM
11H	5‘ CACACCTGCTACTGGTCCTC 3‘ 5‘ TCTGTTTCATGACATGCCTTT 3‘	54°C	10 µM
11C*	5‘ GTGGGTATGCGTGTGTCTCT 3‘ 5‘ ATTAAAGTTGCCACCCTCAC 3‘	58°C	10 µM
6F	5‘ CAACAGCAAAGCACAAAGAA 3‘ 5‘ CAGCAATGCCGATAAGAAAC 3‘	56°C	10 µM
10F *	5‘ TGCGAGTATACCTCTCCTTCA 3‘ 5‘ CAACAACAACAACCGAGAGG 3‘	58°C	10 µM
B6F *	5‘ CACCTCTTGCCTGCACTT 3‘ 5‘ TTTAAACGTCGGTCTGTGTG 3‘	58°C	10 µM
B3H	5‘ GGTATGCGTGGATATGAAGC 3‘ 5‘ CTCGGCATCGCAGTTTC 3‘	58°C	10 µM
AC01R*	5’ ACGTCAGCACACAAACGTC 3’ 5’ CTTCTTCCTGCTTTGCCTCT 3’	58°C	10 µM
AC16R	5’ GGGTGTCGAGGATGAGGT 3’ 5’ TAGTGCCATTAGGGGCTCA 3’	58°C	10 µM

* only red primers will be used in practical session

Table 27: Primer pairs for amplification of microsatellite markers for *Viannia* strains

ALL *VIANNIA* MICROSATELLITE PRIMERS ARE CONFIDENTIAL

C. METHOD PCR:

Step 1: In a sterile 1.5 ml microfuge tube, or the well of a sterile microtiter plate, prepare the Master Mix (MM) as in table 28. The reaction mixtures (MM) should be prepared on ice.

	MM	final concentration
10x PCR buffer (incl. 15 mM $MgCl_2$)	2.5 µl	1x (incl. 1.5 mM $MgCl_2$)
dNTP mix (2.5 mM)	2 µl	200 µM
forward primer (10 µM)	0.5 µl	5 pmol
reverse primer (10 µM)	0.5 µl	5 pmol
Taq (5U/µl)	0.1 µl	1 unit
H_20	17.4 µl	--
Total volume	23 µl	--

Hint: Multiply the quantity shown by the number of samples you have. Always prepare the MM for one sample extra i.e. if you have 5 samples, prepare for 6 so you have MM in excess to meet pipetting errors.

Table 28: Mastermix for microsatellite PCR

Step 2: Label sterile 0.2 ml PCR tubes and dispense 46 µl MM in each tube.

Step 3: Add 2 µL of template DNA to each tube. Prepare **positive control** with 2 µl of reference DNA, and **negative control** with 2 µl ddH_20. The total reaction volume will be 25 µl, vortex and centrifuge the mixture briefly. Overlay (if necessary) the reaction mixtures with 1 drop (50 µl) of sterile light mineral oil to prevent evaporation during the repeated cycles of heating and cooling.

Step 4: Run the following thermocycler programme:

cycle number	denaturation	annealing	extension
1	5 min 95°C	--	--
35	30 sec 95°C	30 sec **AT °C***	1 min 72°C
1	--	--	6 min 72°C
infinite	4°C		

*** AT** = annealing temperature of chosen primerpair as indicated in table 27

Table 29: Amplification scheme for microsatellite PCR

Step 5: Screening of microsatellite variation on MetaPhor agarose gels

- Pour a 4% Metaphor gel (see protocol 2.2).
- Before loading the gels remove the oil from the sample. Go through the oil layer with a pipette tip (with a small air bubble in it) and take out the PCR mix. Wipe your pipette tip with a paper towel to remove the last drop of oil. Alternatively, put the PCR mix with oil on Parafilm and pull

the PCR mix down. The oil will remain on the Parafilm.

- Load the samples (including reference strain) on the gel by mixing 10 µl of the PCR product with 2 µl loading dye. Use a 10 bp ladder in the first and last lane of the gel. Run the gel for 4 hours using 140V (smaller gels may need to be run on lower V).
- Examine the gel by UV light and photograph the gel.
- Compare the sizes of your PCR products.

D. Estimation of microsatellite repeat length using a capillary sequencer:

Separation of fluorescent DNA fragments on automated sequencers, e.g **ABI** or **Beckman Coulter**, is based on gel capillary electrophoresis. The specific softwares, e.g. ABI PRISM GeneMapper (Applied Biosystems, Foster City, CA), provide accurate and high precision sizing of these amplified fragments including automated allele and locus identification.

Step 1: Amplify DNA exactly as described above, but use forward primers labeled with fluorescent dyes. There are three different labels (blue, green and black) available. The size standard will be labeled with a red fluorescent dye. Always include a reference strain in your experiment.

Step 2: Check for PCR products in a 1.2 % agarose gel.

Step 3: Dilute the PCR products in dd H_2O if necessary and add the diluted PCR products to different wells of sterile microtiter plates. Sometimes, in case of weak amplification, it may be necessary to increase the amount of PCR product. This step depends on the age and quality of the labeled primers.

Step 4: The system will calculate the size of DNA fragments by relative comparison to reference peak from the standard. The results will be shown in the form of multiple reference peaks (red) and a test peak (blue, green and/or black).
For the accurate protocol please check with your sequencing unit or company.

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5. Recipes

GENERAL RULE: REAGENTS SHOULD ALWAYS BE OF MOLECULAR BIOLOGY GRADE

1. DNA EXTRACTIONS

0.5 M EDTA (pH 8.0)

1. Molecular weight EDTA =292.25 or 372.24 in the case of the disodium salt
2. Add the needed amount of EDTA to 800 ml of H_2O.
3. Stir vigorously on a magnetic stirrer.
4. As NaOH is soluble at pH 8.0, add NaOH pellets (amount is depending of the type of EDTA used!) to dissolve the EDTA.
5. Check the pH and adjust it to 8.0. Adjust the volume of the solution subsequently to 1 liter with H_2O.
6. Dispense into aliquots and sterilize by autoclaving.

Lysis buffer

1. Weigh 1.46 g NaCl.
2. Add 10 ml EDTA (0.5M, pH 8.0).
3. Add 25 ml Tris.Cl (1 M, pH 7.4).
4. Complete the volume to 500 ml with distilled H2O and aliquot.
5. Sterilize by autoclaving for 15 min at 15 psi by steam autoclave. Store the sterile solution at 4 °C or at room temperature.

5 M NaCl

1. Dissolve 292 g (5 x m.w.) of NaCl in 800 ml of H_2O.
2. Adjust the volume to 1 litre with H_2O.

3. Dispense in aliquots and sterilize by autoclaving.
4. Store the NaCl solution at room temperature.

Phenol

Phenol is commercially purchased for DNA/RNA separation. It is redistilled phenol, chloroform and isoamyl alcohol 25:24:1, saturated with TE-buffer (10mM Tris pH 7.5-8.0; 1mM EDTA-Na_2). Store at 4 ^{o}C.

10% SDS

1. Dissolve 10 g of electrophoresis grade SDS in 90 ml of H2O.
2. Heat to 68 °C and stir with magnetic stirrer to assist dissolution.
3. If necessary, adjust the pH to 7.2 by adding few drops of concentrated HCl.
4. Adjust the volume to 100 ml.
5. Store at room temperature. Sterilization is not necessary. **Do not autoclave!**

Proteinase K (20 mg/ml)

1. Weigh 100 mg lyophilized powder of Proteinase K.
2. Add 5 ml aqua bidest (sterile distilled water).
3. Divide the stock solution into small aliquots and store at –20 °C. Each aliquot can be thawed and frozen several times before discarded.

3 M Na-Acetate (pH 5.2)

1. Dissolve 40.83 g (0.3 x m. w. 136.1) of Na-Acetate in 50-60 ml of H_2O.
2. Adjust the pH to 5.2 with concentrated glacial acetic acid.
3. Adjust the volume to 100 ml with H_2O.
4. Dispense into aliquots and sterilize by autoclaving.

200 ml Tris-EDTA (TE), pH 8.0

1. Pipette 2 ml 1 M Tris-Cl, pH 8.0.
2. Add 400 µl 0.5 M EDTA (pH 8.0).

3. Add 170 ml water.
4. Check the pH and adjust it to 8.0 with 1 M HCl.
5. Complete the volume to 200 ml.
6. Sterilize by autoclaving for 20 minutes at 15 psi on liquid cycle. Store the buffer at room temperature.

1 M Tris-Cl

1. Dissolve 121.1 g (m.w.) of Tris base in 800 ml of H_2O.
2. Adjust the pH to the desired value by adding concentrated HCl slowly under stirring.
3. Allow the solution to cool to room temperature before making final adjustments to the pH. Adjust the volume of the solution to 1 liter with H_2O.
4. Dispense into aliquots and sterilize by autoclaving.
5. If the 1 M solution has a yellow color, discard it and obtain Tris of better quality. The pH of Tris solutions is temperature-dependent and decreases approx. 0.03 pH units for each 1°C increase in temperature. For example, a 0.05 M solution has pH values of 9.5, 8.9, and 8.6 at 5°C, 25°C, and 37°C, respectively.

2. Agarose gel electrophoresis

Tris Borate EDTA (TBE) 10x stock

1. Weigh 108 g Tris.
2. Weigh 55 g boric acid.
3. Add 40 ml 0.5 M EDTA (pH 8.0).
4. Adjust to 1 liter with H_2O.

For gel electrophoresis solution 1x: dilute the stock solution 1:10 in ddH_2O.

For tank electrophoresis solution 0.5x: dilute the stock solution 1:20 in ddH_2O.

TBE is usually made and stored as a 5x or 10x stock solutions. The pH of the concentrated stock buffer should be approx. 8.3. Dilute the concentrated stock buffer just before use and make the gel solution and the electrophoresis buffer from the same concentrated stock solution.

Gel loading buffer

Different solutions are commercially available. If you need to prepare yourself, you can use the following simple recipe:

1. Weigh 30 mg bromophenol blue.
2. Add 15 ml glycerin.
3. Add 6 ml EDTA (0.5 M, pH 8.0).
4. Add 0.3 ml Tris buffer (1 M, pH 8.0).
5. Add up to 30 ml with distilled water .Store at 4 °C.

Ethidium bromide stock (10 mg/ml)

1. Add 100 mg of ethidium bromide to 10 ml of H_2O.
2. Stir on a magnetic stirrer for several hours to ensure that the dye has dissolved.
3. Wrap the container in aluminum foil or transfer the 10 mg/ml solution to a dark bottle and store at 4°C.

Ethidium bromide work solution (0.5 µg/ml)

1. Pipette 100 µl ethidium bromide (10 mg/ml) stock solution
2. Add 2 liter ddH_2O

3. PCR & RFLP

Aliquot all solutions (dNTPs, primer working solutions, buffer, water, , DMSO etc.)

10x amplification buffer

Amplification buffer is commercially available, usually delivered along with *Taq* polymerase. Normally it contains 500 mM KCl, 100 mM Tris-Cl (pH 8.3) and 15 mM $MgCl_2$.

DMSO

Purchase a high grade of DMSO (dimethylsulfoxide, HPLC grade or better). Divide the contents of a fresh bottle into 1 ml aliquots in sterile tubes. Close the tubes tightly and store at room temperature. Use each aliquot only

once and then discard. **Do not autoclave!**

Deoxyribonucleoside triphosphates solution (dNTPs)

dNTPs are commercially available, aliquots should be made and stored at -20°C.

10x restriction enzyme buffer

Specific required incubation buffers are supplied with the restriction enzyme.

6. CAUTIONS

⚠ Chloroform

Chloroform $CHCl_3$ is irritating to the skin, eyes, mucous membranes, and respiratory tract. It is a carcinogen and may damage the liver and kidneys. It is also volatile. Avoid breathing the vapors. Wear appropriate gloves and safety glasses. Always use in a chemical fume hood.

⚠ Phenol

Phenol is extremely toxic, highly corrosive, and can cause severe burns. It may be harmful by inhalation, ingestion, or skin absorption. Wear appropriate gloves, goggles, and protective clothing. Always use in a chemical fume hood. Rinse any areas of skin that come in contact with phenol with a large volume of water and wash with soap and water; do not use ethanol!

⚠ SDS (sodium dodecyl sulphate)

SDS is toxic, an irritant, and poses a risk of severe damage to the eyes. It may be harmful by inhalation, ingestion, or skin absorption. Wear appropriate gloves and safety goggles. Do not breathe the dust.

⚠ Ethidium bromide

Ethidium bromide is a powerful mutagen and is toxic. Consult the local institutional safety officer for specific handling and disposal procedures. Avoid breathing the dust. Wear appropriate gloves when working with solutions that contain this dye.

⚠ Sodium hydroxide (NaOH)

Highly toxic and caustic and should be handled with great care. Wear appropriate gloves and a face mask. All concentrated bases should be handled in a similar manner.

⚠ DMSO (dimetylsulfoxide)

DMSO may be harmful by inhalation or skin absorption. Wear appropriate gloves and safety glasses. Use in

a chemical fume hood. DMSO is also combustible. Store in a tightly closed container. Keep away from heat, sparks, and open flame.

⚠ Acrylamide (unpolymerised)

Is a potent neurotoxin and is absorbed through skin (effects are cumulative). Use gloves and a face mask when weighing.

⚠ Polyacrylamide

Is considered nontoxic, but it should be handled with care because it might contain small quantities of unpolymerised acrylamide.

⚠ Ammonium persulfate

Ammonium persulfate is extremely destructive to tissue of the mucous membranes and upper respiratory tract, eyes and skin. Inhalation may be fatal. Wear appropriate gloves, safety glasses, and protective clothing. Always use in a chemical fume hood. Wash thoroughly after handling.

⚠ KOH and KOH/methanol

KOH and KOH/methanol can be highly toxic. It may be harmful by inhalation, ingestion, or skin absorption. Solutions are caustic and should be handled with great care. Wear appropriate gloves.

⚠ TEMED (N,N,N',N'-tetramethylethylenediamine)

TEMED is extremely destructive to tissues of the mucous membranes and upper respiratory tract, eyes and skin. Inhalation may be fatal. Prolonged contact can cause severe irritation or burns. Wear appropriate gloves, safety glasses, and other protective clothing. Use only in a chemical fume hood. Wash thoroughly after handling. It is flammable; vapour may travel a considerable distance to source of ignition and flash back. Keep away from heat, sparks, and open flame.

⚠ Nitric acid (HNO_3)

Is volatile and must be handled with great care. It is toxic by inhalation, ingestion, and skin absorption. Wear appropriate gloves and safety goggles. Use a chemical fume hood. Do not breathe the vapours. Keep away from heat, spark, and open flame.

⚠ Silver nitrate ($AgNO_3$)

Strong oxidising agent and should be handled with care. It may be harmful by inhalation, ingestion, or skin absorption. Avoid contact with skin. Wear appropriate gloves and safety glasses. It can cause explosion upon contact with other materials.

⚠ Formaldehyde (HCOH)

Is highly toxic and volatile. It is also a carcinogen. It is readily absorbed through the skin and is irritating or destructive to the skin, eyes, mucous membranes and upper respiratory tract. Avoid breathing the vapours. Wear appropriate gloves and safety glasses. Always use in a chemical fume hood. Keep away from heat, sparks and open flame.

⚠ Acetic acid (concentrated)

Must be handled with great care. It may be harmful by inhalation, ingestion, or skin absorption. Wear appropriate gloves and goggles. Use in a chemical fume hood.

USEFUL WEBSITES:

http://www.bio.davidson.edu/courses/Molbio/tips/funDNAgel.html

http://www.protocol-online.org/

GENERAL REFERENCES:

Sambrook, J and Russel, D. W. Molecular cloning: A laboratory manual. (2001). 3rd Edition.Cold Spring Harbor Laboratory Press, New York: 8.22, book 2