# Protocols for handling and working with *Leishmania* species

# (*Leishmania* Protocols for Dummies)

**Table of Contents**

1 Culture of *Leishmania sp.* ..... 3
1.1 Passage of cultures ..... 3
1.2 Stabilates ..... 4
1.3 Counting ..... 4
1.4 Making growth curves ..... 5
1.5 Preparation of *L. major* metacyclics by agglutination ..... 7
1.6 Percoll gradient purification of metacyclics ..... 7
1.7 Preparation of axenic amastigotes ..... 8
1.8 Percoll gradient purification of amastigotes from infected macrophages ..... 9
1.9 Purification of amastigotes from infected tissues ..... 10
2 DNA preparation from *Leishmania sp.* ..... 11
3 Preparation of protein extracts from *Leishmania sp.* ..... 12
3.1 Cell extract ..... 12
3.2 Culture supernatant protein ..... 12
4 Immunofluorescence ..... 13
4.1 Fixation ..... 13
4.2 Immunofluorescence ..... 13
5 Transfection ..... 14
5.1 AMAXA 2D Method ..... 14
5.2 AMAXA 4D Method ..... 14
5.3 Dilution series and selecting clones ..... 15
6 Phenotypic analysis of mutant cell lines ..... 16
6.1 Infection of macrophages and drugging protocol ..... 16
7 Viability assays ..... 17
8 Luciferase reporter gene assay ..... 18
9 Species identification ..... 19
9.1 PCR ..... 19
9.2 Restriction digests ..... 19
Appendix A: Molecular analysis of *Leishmania* mutants ..... 21
A1 PCR ..... 21
A2 Southern blot ..... 23
A3 Western blot ..... 25

# 1 Culture of *Leishmania sp.*

*Leishmania* work should only be carried out in the Containment Level 3 laboratories.
*Leishmania* transported outside of the tissue culture room need to be in double containment within a metal container.
Tissue culture-specific lab coats and gloves should be worn at all times.
Hoods should be disinfected with 70% ethanol prior to use and everything to go into the hood should first be sprayed with 70% ethanol.
Waste plastics should be soaked in trigene overnight then placed in the autoclavable waste bag inside a metal bin.
Spillages should be cleaned with 70% ethanol or 10% trigene.

See: SOP001 CL3 procedure for entering and exit; SOP002 CL emergency procedures; SOP003 Fumigation procedure of CL3 labs; SOP006 CL3 waste material sterilisation and laundry; SOP010 Safety cabinet monthly cleaning; SOP101 Storage of Leishmania; SOP102 Safe handling of Leishmania

## 1.1 Passage of cultures

Most promastigote cultures grow in HOMEM supplemented with 10% heat-inactivated foetal calf serum (FCS) and 1% penicillin/streptomycin solution.

- 500 ml HOMEM
- 50 ml FCS
- 5 ml penicillin/streptomycin solution

Grow in an airtight flask (non-vented) and incubate upright in a 25°C incubator.
For faster growth, incubate the flask horizontally, this increases the oxygenation within the medium.
Parasites need to be passaged every 2-3 days, typically a 1:20 dilution of the old culture with fresh medium, although take into consideration any growth defects.

**IMPORTANT:** Promastigotes lose infectivity through serial passaging. As a general rule, keep the passage number under 20.

**Table 1.** Guideline concentrations for antibiotic selection

| **Resistance cassette** | **Antibiotic** | **Supplier** | **Concentration** |
|---|---|---|---|
| BLAST | Blasticidin | Invivogen | 10-20 µg $ml^{-1}$ |
| HYG | Hygromycin | Invivogen | 50 µg $ml^{-1}$ |
| BLE | Bleomycin | Invivogen | 10 µg $ml^{-1}$ |
| NEO | G418 neocyin | Invivogen | 50 µg $ml^{-1}$ |
| PAC | Puromycin | Invivogen | 50 µg $ml^{-1}$, 75 µg/ml (L.mex) |

| | | | |
|---|---|---|---|
| SAT | LEXSY NTC nourseothricin | Jena Bioscience | 25 µg $ml^{-1}$, 100 µg $ml^{-1}$ (for L.mj), 75 µg/ml (L.mex) |

# 1.2 Stabilates

## *1.2.1 Preparation of stabilates*

For 1 stabilate (1 ml):

- 500 µl log-phase culture
- 450 µl HOMEM supplemented with 20% FCS and 1% penicillin/streptomycin solution
- 50 µl DMSO (cryopreservative) (final concentration: 5%)

Place cryovials in an insulated container (e.g. a tip box with cotton wool) and place in -80$^{o}$C freezer overnight.
Insulation allows gradual temperature reduction which ensures maximum survival of cells during freezing process.
Transfer cryovials to liquid nitrogen for long-term storage.

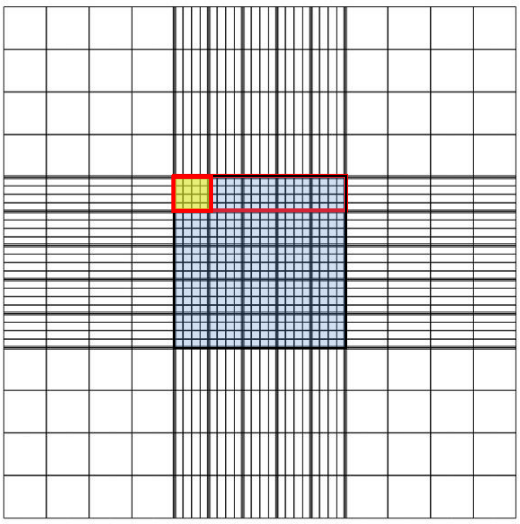
## *1.2.2 Recovery of stabilates*

Transport cryovials from liquid nitrogen storage to the tissue culture hood in double containment.
Defrost the cryovials rapidly by warming in your gloved hands.
Pipette stabilate into flasks containing prewarmed HOMEM/20% FCS as soon as possible.

**IMPORTANT:** Rupturing of cryovials may occur when opening.

# 1.3 Counting

Mix 20 µl of cells from culture with 20 µl of 2% formaldehyde for a dilution factor of 2.
May need to dilute 1 in 5 or 1 in 10 if the culture is very dense.
Place 10 µl of the fixed cells on the Neubauer haemocytometer and leave at RT for 5 min to allow the cells to settle.
Count 3 rows of 5 squares (highlighted in red), add the counts together and divide by 3 to get the average of 5 small squares.
Multiply the number by $10^5$ to give the number of cells in 1 ml of undiluted culture.



**How does this calculation work?**

Each small square (highlighted in yellow) = 0.2 x 0.2 mm = 0.04 $mm^2$ containing 4 nL
Thus cell count per nL = (average cell count per small square / 4 nL)
1 mL = 1 x $10^6$ nL
Thus cell count per ml = (average cell count per small square / 4 nL) x $10^6$
So if you diluted your sample 2x and counted 5 small squares (ie 1 row) then:
Cells/ml = ((Total cell count in 5 small squares / 5) x 2)/ 4 nL) x $10^6$

Each larger block (blue) = 1x1 mm = 1 $mm^2$ containing 100 nL

Thus cell count per nL = (average cell count per larger block / 100 nL)

1 mL = 1 x $10^6$ nL

Thus cell count per ml = (average cell count per larger block / 100 nL) x $10^6$

So if you diluted your sample 2x and counted 1 larger block then:

Cells/ml = ((Total cell count in a large block x 2)/ 100 nL)) x $10^6$

## 1.4 Making growth curves

Inoculate 10 ml HOMEM/10% FCS with $1\times10^5$ *Leishmania* cells (log-phase culture) per ml of medium, i.e. $1\times10^6$ *Leishmania* cells for 10 ml of medium.
Count the cells once a day (preferably at the same time) until the cells stop growing, i.e. reach stationary phase.

*Leishmania* promastigote life cycle stages:
Mid log phase: ~ $5\times10^6$ cells/ml
Late log phase: ~ $1\times10^7$ cells/ml
Stationary phase: $2.5\times10^7$ cells/ml (day 6 on the curve on the following page)

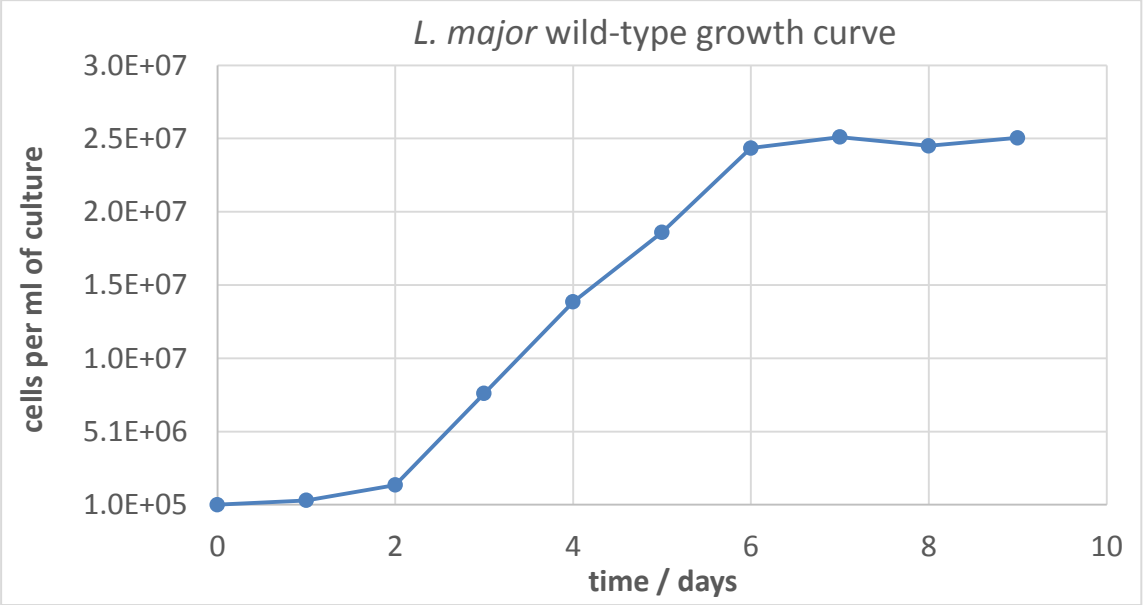
In the late log phase and stationary phase, procyclics differentiate into metacyclics. These cells are thinner, more elongated and have a long flagellum. They are also highly motile compared to procyclics but they do not divide.

**Table 2.** Taken from **Rogers et al. (20012) Parasitology; 124(Pt 5):495-507**

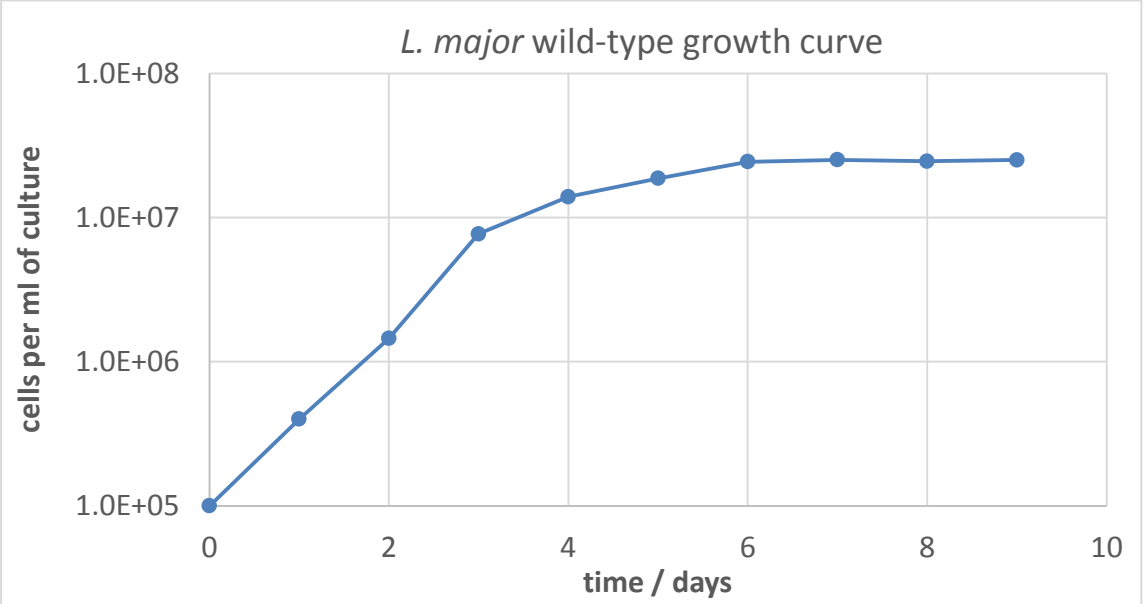
| Morphological category | Criteria | Illustration |
|---|---|---|
| Amastigote | Ovoid body form, no flagellum protruding from flagellar pocket |  |
| Procyclic promastigote | Body length 6·5 – 11·5 $\mu$m, flagellum < body length (body width variable) |  |
| Nectomonad promastigote | Body length ≥ 12 $\mu$m, (body width and flagellar length variable) |  |
| Leptomonad promastigote | Body length 6·5 – 11·5 $\mu$m, flagellum ≥ body length (body width variable) |  |
| Haptomonad promastigote | Disc-like expansion of flagellar tip (body form and flagellar length variable) |  |
| Metacyclic promastigote | Body length ≤ 8 $\mu$m, body width ≤ 1·0 $\mu$m, flagellum > body length |  |
| Paramastigote | Kinetoplast adjacent tonucleus, external flagellum present |  |

**IMPORTANT:** The cell density of a given cell line may not reach $2.5\times10^7$ cells/ml. This is especially true if antibiotics are present in the medium, or if the cell line has been in culture for a long time. Nevertheless, the cell density should reach $1\times10^7$ cells/ml from $1\times10^5$ cells/ml. If not, the cell line has a growth defect.

A typical growth curve will have a sigmoidal shape:




The same curve with a logarithmic scale on the Y axis shows the log growth until around $10^7$ cells/ml. The cells subsequently carry on growing at a much lower rate until they reach the stationary phase ~$2.5\times10^7$ cells/ml.



## 1.5 Preparation of *L. major* metacyclics by agglutination

During the transformation of *L. major* promastigotes to metacyclics there is a change in cell surface molecules. *L. major* promastigotes are agglutinated by peanut lectin whereas metacyclics are not. This technique does not work with *L. mexicana* as the cell surface molecules are different. (**da Silva and Sacks (1987) Infect Immun; 55(11):2802-2806**)

### *1.5.1 Quick count of metacyclics in a culture*

- Take 200 µl of a stationary phase culture (3 days stationary).
- Add 10 µl 1 mg/ml peanut lectin.
- Incubate 15-20 min at RT.
- Centrifuge at 100 x *g* (lowest possible setting on the centrifuge) for 3-5 min.
- Take 20 µl of supernatant and add 20 µl of 2% formaldehyde.
- Count metacyclic promastigotes.

### *1.5.2 Larger scale purification*

- Centrifuge a 10 ml stationary phase (3 days stationary) *L. major* culture at 1000 x *g* for 10 min.
  - *A stationary phase L.major culture usually contains ~20% metacyclics, so start with 5x more than you need*
- Remove 9 ml of medium and resuspend the cells in the 1 ml of medium remaining in the tube.
- Add 50 µl of 1 mg/ml peanut lectin per 1 ml (50 µg/ml final).
- Incubate 15-20 min at RT.
- Centrifuge at 100 x *g* (lowest possible setting on the centrifuge) for 5 min.
- Remove the supernatant containing the metacyclics without disturbing the agglutinated promastigote pellet and transfer to a new eppendorf.
- Wash the metacyclics in PBS.

**IMPORTANT:** Metacyclics do not like to stay in PBS for very long. They will show significant decrease in motility after 30 min. It is better to keep the metacyclics in the medium following agglutination and only washing them in PBS prior to use. If using for *in vivo* infection, check the condition of the parasites after infection.

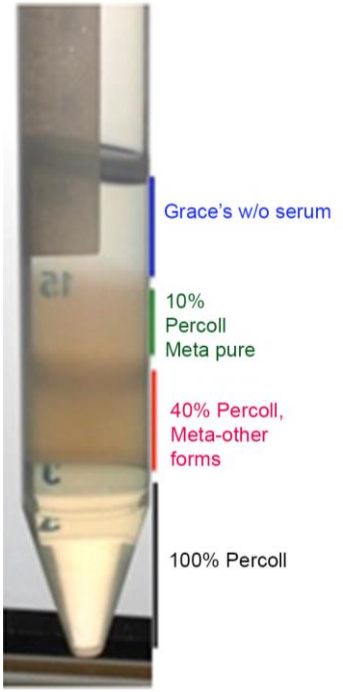
## 1.6 Percoll gradient purification of metacyclics

Before you begin:

- Prepare a stock of 1.5 M NaCl or 10x concentrated Grace´s media without serum.
- Filter the Percoll.
- Prepare the Stock Isotonic Percoll (SIP) 100%: Add 9 parts of Percoll to 1 part of 1.5 M NaCl or 10x media to make an isotonic Percoll solution.
- 40% Percoll: Dilute 40% SIP with 60% 0.15 M NaCl or 1x media.
- 10% Percoll: Dilute 10% SIP with 90% 0.15 M NaCl or 1x media.
- Make the gradient adding the 100/40/10% Percoll carefully to the wall of the falcon tube - typically using 3 ml of each for $1 \times 10^8$ parasites.

- Grow parasites to stationary phase in Grace´s media.

- Centrifuge $1\times10^8$ parasites at 3000 rpm for 10 min and resuspend in 3 ml incomplete Grace's (no serum).
- Add the parasites on top of the 10% Percoll layer.
- Centrifuge 1300 x *g* for 10 min.
- Transfer 10% layer (meta pure) to a new tube and wash twice in PBS.




## 1.7 Preparation of axenic amastigotes

**1.** Method for *L. mexicana* (**Bates et al., (1992) Parasitology; 105(Pt 2):193-202**)
The medium is as follows:
For 500ml:

- 400 ml Schneider's Drosophila Medium
- 100 ml HIFCS
- 3 ml HEMIN (2.5 mg/ml in 50 mM NaOH)

Final pH 5.5 adjusted with HCl and filter-sterilised.

To prepare *L. mexicana* axenic amastigotes:

- Take a late log phase culture of *L. mexicana*.
- Centrifuge at 1000 x *g* for 10 min.
- Remove the supernatant.
- Subculture into the amastigote medium at $1\times10^6$ cells/ml.
- Incubate at 32.5°C, 5% $CO_2$ in vented cap flasks.
- Subculture weekly.
- To count the parasites, they must be slowly passed through a 26 gauge needle around 10 times to separate clumps but be careful not to disrupt the cells.

**2.** Method for *L. infantum*
The medium is as follows:

- 8 ml M199 medium

- 1 ml 100 mM sodium phosphate buffer pH 5.5
- 1 ml FCS

➢ Take a stationary phase culture of *L. mexicana* or *L. infantum*.
➢ Centrifuge at 1000 x *g* for 10 min.
➢ Remove the supernatant.
➢ Wash the cells in 10 ml sterile PBS.
➢ Centrifuge at 1000 x *g* for 10 min.
➢ Remove the supernatant and recover the cells in 10 ml amastigote culture medium.
➢ Incubate for 3 days at 35°C, 5% $CO_2$ in vented cap flasks (place on its side).

## 1.8 Percoll gradient purification of amastigotes from infected macrophages

**Chang (1980) Science 1980; 209(4462):1240–1242**

Before you begin:
- Prepare a stock of 1.5 M NaCl or 10x concentrated Grace´s media without serum.
- Prepare the Stock Isotonic Percoll (SIP) 100%: Add 9 parts of Percoll to 1 part of 1.5 M NaCl or 10x media to make an isotonic Percoll solution. Better results are obtained when using 1.5 M NaCl.
- 45% Percoll: Dilute 45% SIP with 55% 1.5 M NaCl or 1x media - typically prepare 10 ml [4.5 ml SIP and 5.5 ml of NaCl or media].

**Large scale macrophage infections**

➢ Grow parasites to stationary phase in Grace´s media for metacyclic-enriched cultures (6-7 d post-inoculation with $1\times10^5$ log culture in Grace's media).
➢ Centrifuge 3000 rpm for 10 min.
➢ Optional: Serum-opsonise metacyclics by resuspending $1\times10^8$ metacyclics in 7.5 ml pre-warmed Grace's media without serum + 25% (2.5 ml) human serum (or any non-inactivated serum) and incubate at 37°C for 30 min, shaking the tubes every 5 min.
➢ Centrifuge and resuspend the parasites in 5 ml DMEM/10% FCS-HI.
➢ Infect macrophages at 1:15 ratio with purified metacyclics in 100 mm cell culture dishes.
➢ Incubate at 37°C for ~6-8 h.
➢ Wash 2x with PBS and add fresh DMEM media.
➢ Incubate for a further 24 h.

**Amastigote separation using 45/100% Percoll gradient or 45% *in situ* gradient**

➢ Remove most of the DMEM media, leaving a small volume (~5 ml) to scrape the infected macrophages cells.
➢ Collect the cells into a 50 ml falcon tube, up to a total of 6-8 culture dishes per tube ($1\times10^7$ macrophages per dish) - Use no more than $1\times10^8$ macrophages per 15 ml 45% Percoll.
➢ Centrifuge 3500 x *g* for 10 min.
➢ Resuspend the cells in 8 ml PBS + anti-protease inhibitors.
➢ Pass the volume through a syringe coupled to a 25G (or 27G) needle ten times. Check under the microscope for the correct lysis of the cells.

- Centrifuge 3500 x *g* for 10 min.
- Resuspend the pellet in 45% Percoll (15 ml per 6-8 culture dishes) and either:

  **a. *In situ* gradient – Bigger yields**

  Centrifuge 3500 x *g* for 35 min. Remove carefully the top layer containing macrophages and collect all the remaining volume containing amastigotes.

  **b. 45/100% Percoll gradient – Smaller but cleaner yields**

  Layer the 45% solution over a cushion of 15 ml 100% Percoll. Centrifuge 3500 x *g* for 35 min. Remove carefully the top layer containing macrophages and collect the 45%-100% interphase containing pure amastigotes.
- Dilute and wash the Percoll solution containing parasites in 50 ml falcon tube with PBS or media without serum, mix well and centrifuge at 3500 x *g* for 10 min. Remove any foam or macrophages that may still be forming a thin layer at the top. The pellet will be the pure amastigotes.

## 1.9 Purification of amastigotes from infected tissues

### *1.9.1 Quick purification of amastigotes*

Suitable for re-infection of animals.

### *1.9.2 Larger scale purification*

To obtain high numbers of amastigotes for further experiments a protocol based on **Hart et al. (1981) Parasitology; 82(Pt 3):345-355** can be used.

- Remove mouse organs/tissues and cut into small pieces.
- Transfer to a Dounce glass tissue grinder with 400 ml PSGEMKA buffer until no large pieces remain.
- Resuspend 50 mg saponin in 1 ml PSGEMKA buffer and add to the 400 ml crude suspension.
- Centrifuge the crude suspension at 3400 rpm for 10 min.
- Discard the supernatant.
- Wash the pellet three times in PSGEMKA buffer.
- Resuspend the pellet in a final volume of 100 ml PSGEMKA buffer.
- Prepare a column with glass wool and a 100 ml sephadex solution (2.5 g sephadex CM25 swelled in 100 ml PSGEMKA at 4 °C overnight).
- Pipette the 100 ml amastigote suspension into the column.
- Discard the first 100 ml of flow-through.
- Collect the subsequent volume as the amastigote preparation.
- Centrifuge the amastigote preparation at 3400 rpm for 10 min.
- Wash the pellet three times in PSGEMKA buffer.

PSGEMKA: 20 mM sodium phosphate, 104 mM sodium chloride, 5.5 mM D-glucose, 0.5 mM EDTA, 10 mM magnesium chloride, 10 mM potassium chloride and 0.02% (w/v) BSA

# 2 DNA preparation from *Leishmania sp.*

- Start with a 10 ml culture.
- Pellet the cells by centrifugation at 1000 x *g* for 10 min.
- Resuspend the pellet in 0.5 ml TELT and add 10 µl RNase A (10 mg/ml).
- Incubate at RT for 5 min.
- Extract twice with phenol: add 0.5 ml phenol, vortex, centrifuge at top speed for 5 min, then recover the top aqueous phase.
- Extract twice with chloroform/isoamyl alcohol (24:1).
- Add 1 ml ice-cold ethanol.
- Centrifuge at top speed for 30 min at 4°C.
- Wash the pellet in 75% ethanol.
- Dry and resuspend in 50 µl TE.

TELT: 50 mM Tris Cl pH 8.0, 62.5 mM EDTA, 2.5 M LiCl, 4% Triton X-100
**IMPORTANT:** TELT preps can be difficult to digest with some restriction enzymes.

***Alternatively, use the DNeasy Tissue kit (Qiagen) using 10 ml culture.

# 3 Preparation of protein extracts from *Leishmania sp.*

## 3.1 Cell extract

- Start with a 10 ml culture.
- Centrifuge at 1000 x *g* for 10 min.
- Remove the supernatant.
- Resuspend the cells in 10 ml PBS.
- Centrifuge at 1000 x *g* for 10 min.
- Lyse the cell pellet in 2% SDS or 1x SDS-PAGE loading buffer and boil for 5 min.
- Store at -20°C.

Ideally the cells should be resuspended in 1x SDS-PAGE loading buffer at $10^7$ cells/10 µl and boiled 5 min. For a SDS-PAGE gel we usually run $5\times10^6$ or $10^7$ cells per well i.e. 5 to 10 µl.

**NOTE:** Quantification of protein is possible in 2% SDS lysate as it is compatible with the Pierce BCA protein assay. But it is not possible when using the 1x SDS-PAGE loading buffer as it contains mercaptoethanol or DTT, which is not compatible with the protein assay kit.
**IMPORTANT:** For specific preparations, the use of a more appropriate lysis buffer and the addition of peptidase inhibitors may be required.

## 3.2 Culture supernatant protein

- Take the supernatant from the centrifugation step above.
- Filter through a 0.2 µm filter to remove any cells from the sample.
- Take 1 ml of filtered supernatant and add 250 µl of ice cold 0.5 mg/ml TCA.
- Incubate on ice for 10 min.
- Centrifuge at top speed for 5 min.
- Wash twice in ice cold acetone.
- Resuspend the protein pellet in 1× SDS-PAGE loading buffer (1/10 of the original volume).

Alternative method:

- Cool acetone to -20$^{o}$C.
- Filter the supernatant.
- Add 1 ml of ice cold acetone to 250 µl of filtered supernatant.
- Vortex and incubate at -20$^{o}$C for 1 h.
- Centrifuge at 13000 x *g* for 10 min.
- Resuspend the protein pellet in 1× SDS-PAGE loading buffer (1/10 of the original volume).

**IMPORTANT:** 10 µl of the supernatant on a SDS-PAGE shows a large band around 60 kDa (most likely BSA). When concentrated, this protein can affect the running of others bands (distorted gel). Alternatively, cells can be grown at a high concentration (i.e. $10^8$ cells/ml in 10 ml) for a short amount of time (3-4 h) in serum-less medium to get rid of the protein contents of the serum and the medium can be analysed as above.

# 4 Immunofluorescence

## 4.1 Fixation

- Start with 200 µl of mid-log cells.
- Wash twice in PBS and resuspend in 100 µl PBS.
- Fix in 1% methanol-free formaldehyde (2% stock).
- Incubate at RT for 30 min.
- Add Triton X-100 to 0.1% final (1% stock).
- Incubate at RT for 10 min.
- Add glycine to 0.1 M (1M stock).
- Incubate at RT for 10 min.
- Centrifuge at 2000 rpm for 10 min and resuspend in 200 to 400 µl PBS.
- Spread on slide (50 µl drop in the centre).
- Dry completely at RT.

**IMPORTANT:** Wash the slides with ethanol prior to use, dirty slides have a high fluorescence background.

## 4.2 Immunofluorescence

- Rehydrate the cells in PBS at RT for 5 min (optional).
- Block the slide with TB at RT for 5 min.
- Add the TB-diluted primary antibody to the slide.
- Incubate 1 h at RT (incubation time can be increased up to overnight if slides are kept at 4°C, as for some antibodies it can increase the signal strength and specificity).
- Wash 3x in PBS.
- Add the TB-diluted secondary antibody to the slide.
- Incubate 1 h at RT in the dark.
- Wash 3x in PBS.
- Dry and mount.

**IMPORTANT:** Slides must NOT be allowed to dry completely, as this will cause high background.

TB: PBS with 0.1% Triton X-100, 0.1% BSA

Mounting solution: 50% glycerol, 2.5% DABCO, DAPI 1 µg/ml (alternatively, DAPI can be incubated for only 5 min prior to the final washes, it helps reducing the background signal).

# 5 Transfection

Before you begin:

- DNA for homologous recombination:

Start with at least 100 µg and cut with the appropriate enzyme(s) (at least two sequential digestions with the right units of enzymes, in a large volume i.e. 200 µl).
Gel-extract the fragment to increase the chances of removing any uncut DNA – Use 5 or 6 columns as each column only binds up to 10 µg of DNA.

- DNA should be ethanol or isopropanol precipitated, air-dried in the hood then resuspended in a small volume of sterile $dH_2O$.

Do not allow the pellet to overdry as it becomes almost impossible to get back into solution.

- Ensure the parasites will be in mid-log phase (0.8-1.0x$10^6$ cells/ml) and you will have sufficient cells to perform a control transfection (using sterile water) for each antibiotic you use for selection.

## 5.1 AMAXA 2D Method

This method uses the Amaxa Nucleofector System (http://www.amaxa.com/) customised for the Human T Cell Nucleofector Kit.

- For one transfection, pellet 5x$10^7$ log-phase cells at 1000 x *g* for 10 min.
- Resuspend the pellet in 100 µl of Human T Cell Nucleofector Solution (prepared previously according to manufacturer's instructions) and transfer to the cuvette.
- Add approximately 10 µg DNA (see Appendix B), ideally in a volume of about 10 µl.
- Flick the cuvette gently to mix.
- Ensure the cuvette electrodes are dry and place in the 2D Nucleofector.
- Use program U-033 to transfect the cells (alternatively program V-033 can be used, although it is a bit harsher, it should be higher efficiency according to the manufacturer).
- Transfer transfected cells to 10 ml HOMEM with 10% FCS.
- Immediately split the culture into 2 separate flasks (5 ml each - to select for independent integration events. This is not necessary for episome transfection)
- Allow the cells to recover overnight in 25°C incubator.
- Select cells with appropriate antibiotics and plate out dilutions for clones (see Section 5.3).

## 5.2 AMAXA 4D Method

This method uses the P3 Primary cell 4D-Nucleofector® X kit (Cat no. V4XP-3032).

- Harvest 8x$10^6$ cells at 1000 x *g* at RT for 10 min.
- Remove supernatant and wash pellet twice in 1x PBS to remove all medium.
- Re-suspend the pellet in **20 µl** of P3 Nucleofector solution at 4°C.
- Add **2 µl of 1 µg/µl** of linearised DNA into the suspension and transfect immediately using program FI-115.
- For recovery after transfection:
  - Allow strip to stand at RT for 2 min.
  - Then add **80 µl** of medium and incubate at 25°C for 10 min.
  - Resuspend the transfected cells into 10 ml of medium and incubate overnight at 25°C.

## 5.3 Dilution series and selecting clones

**NOTE:** If transfecting with an episome and/or don't need to clone out cells, you should dilute the overnight transfection culture at least 1:4 with medium containing appropriate antibiotics.

### *5.3.1 96 well plate serial dilution*

The general method that should be used involves dilution of the overnight recovered cells based on the expected transfection efficiency. For example, for an AMAXA transfection of a pRIB construct the expected transfection efficiency is about $2x10^{-5}$/µg DNA. Therefore you would expect transfecting $5x10^7$ *Leishmania* with 10 µg of DNA to yield:

$$\frac{5 \times 10^7}{2 \times 10^{-5} \times 10}$$

= 2500 clones in total 10ml

= 1000 clones per 4 ml of transfection as diluted and plated below.

Integration into the ribosomal locus using the pRIB construct is a very efficient due to the high number of integration sites, however, other integrations such as 1st and particularly 2nd allele knock-outs have far lower efficiency.

The following dilution series works well for most transfections.

- Add antibiotics directly to the flask of transfected cells.
- Add 4 ml of cells from each flask to a Falcon tube containing 20 ml HOMEM/FCS. Mix well by inversion.
- Add 2 ml of this to 22 ml HOMEM/FCS in second Falcon Tube.
- Take 2 ml of the second dilution and add to 22 ml of HOMEM/FCS in a third Falcon tube.

Plate the contents of each Falcon tube out on a 96 well microplate at 200 µl per well (1 plate per Falcon tube dilution).

- Incubate at 25°C for 3-4 wk.
- To the remaining 1 ml in each flask, add fresh HOMEM/FCS with the appropriate antibiotics to prepare a population of transfectants.
- Do the same with 1 ml of each control transfection culture to ensure the antibiotic selection is working as the cells should die off.

**NOTE:** Should no clones grow on the dilution plates it is possible to plate out the population to obtain one clone from each flask.

### *5.3.2 Petri dishes*

This method guarantees clonal cell lines but does not always work.

Prepare agar plates as follows:

- 8 ml of 2x HOMEM
- 4 ml FCS
- 10 ml melted 2% agar (prepared with distilled water and autoclaved)

- Spin the transfected cells and resuspend in 100 µl medium.
- Spread on the agar plate. Do not apply too much pressure as it is a soft agar plate.
- Seal with Parafilm and incubate upside down at 25°C for 3-4 wk.
- Select colonies, which are small and white, like bacterial colonies.

# 6 Phenotypic analysis of mutant cell lines

**1.** Growth curve
**2.** Differentiation into metacyclics: peanut lectin, western blot for SHERP and HASP
**3.** Macrophage infection *in vitro*

## 6.1 Infection of macrophages and drugging protocol

Before you begin, need to know how many slides/wells are needed – as slides are needed to check the infection before the drugging protocol.

**Day 1 - Plate out macrophages**

- Centrifuge macrophages at 1000-1500 rpm for 10 min at 4°C, resuspend in fresh medium and count.
- Dilute macrophages at $5x10^5$ cells/ml with RPMI 1660 + 10% HIFCS + 0.5% Gentamicin.
- Pipette 100 µl of the macrophage suspension into the wells of a 16-well Lab-Tek tissue culture slides ($5x10^4$ macrophages/well).
- Incubate slides at 37°C, 5% $CO_2$ for 24 h.

**Day 2 - Infect macrophages with cultured promastigotes (or amastigotes)**

- Stationary phase promastigotes are counted using a haemocytometer and diluted to the relevant concentration for desired ratio in RPMI 1640 + 10% HIFCS then 100 µl is added to the wells.
- Axenic amastigotes are separated by passing through a 26G needle and counted using a haemocytometer, then diluted to the relevant concentration for desired ratio and 100 µl is added to the wells.

- Slides are incubated for a further 24 h at 32°C (*L. mexicana)*, or 37°C (*L. major, L. donovani, L. infantum*) with 5% $CO_2$ for the infection.

**Day 3 - Check the infection and start the drugging protocol**

- To check the level of infection is suitably high to proceed with the drugging, remove the medium, fix with 100% methanol for <1 min, and stain with 10% Giemsa for 10 min.
- To the slides for the drugging protocol, remove the medium, wash several times with the medium, and then add 200 µl fresh RPMI/10% FCS with appropriate drugs in serial dilution. Infection control wells are recovered with 200 µl RPMI/10% FCS only.
- If only testing infectivity of parasites, cells are simply recovered with RPMI/10% FCS.

**Day 6** stop of 3 day experiment, or 2nd drugging of 5 day experiment

**Day 8** stop of 5 day experiment, or 3rd drugging for 7 day experiment

**Day 10** 7 day stop

When drugging is repeated, slides are first washed with RPMI several times.
When stopped, slides are fixed with 100% methanol and stained with 10% Giemsa for 10 min.
Slides are then counted for % of macrophages infected and the number of parasites per macrophage.

# 7 Viability assays

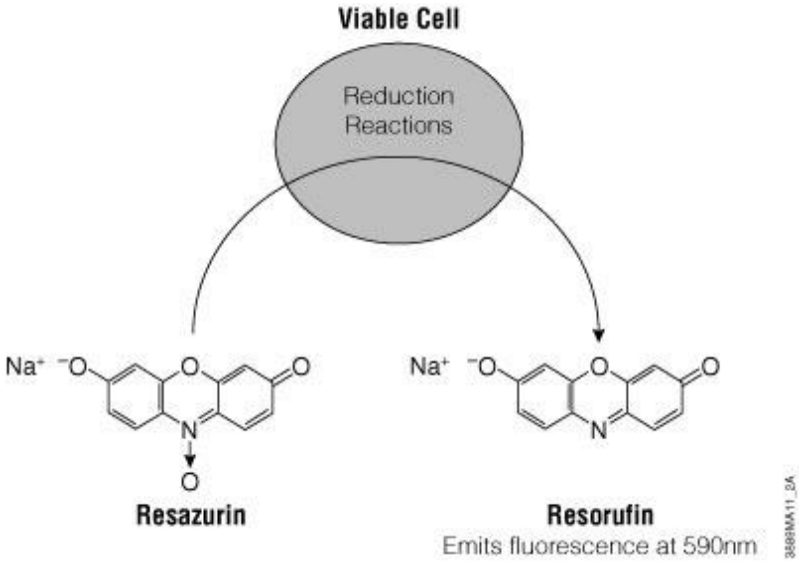



**Figure.** Reduction of resazurin to resorufin in living cells.

To check cell viability in drug screening, the resazurin (Alamar Blue) assay can be used.

Before you begin, prepare a stock solution of 12.5 mg resazurin dissolved in 100 ml distilled water and filter-sterilised.
**IMPORTANT:** Keep resazurin out of direct light. Keep resazurin and assay plates covered.

- Count cells, promastigotes or amastigotes, and prepare cell suspension to $10^6$ cells/ml in appropriate medium.
- To a 96 well plate, add 50 µl of drug diluted in appropriate medium.
- Add 50 µl of the cell suspension.
- Include controls:
  - Cell growth control – Cells with medium and without drug
  - Fluorescence from medium – Medium only and medium with 1% DMSO
- Incubate 48 h at either 25°C or 35°C for promastigotes and amastigotes respectively.
- Add 20 µl of resazurin to each well and incubate the plates for another 24 h.
- Read the plates at 590 nm.

# 8 Luciferase reporter gene assay

**IMPORTANT:** The bioluminescent signal can vary depending on the growth phase used. When selecting the ´highest expressing clones´ for *in vivo* experiments, the bioluminescent signal of the stage used for infection, i.e. stationary phase or purified metacyclic promastigotes (**section 1.5**), should also be checked. The reporter assay can also be performed using amastigotes purified from infected tissues (**section 1.7**).

Use the substrate from the Luciferase Reporter Gene Assay (Roche) or the *in vivo* substrate at 1:100 dilution.

- Count cells and centrifuge sufficient cells to perform in duplicate at 1000 x *g* for 10 min.
- Wash in PBS or phenol red-free RPMI 1640 (PAA).
- Resuspend in PBS or phenol red-free RPMI at the required number of cells per 100 µl.
- Add the cell suspensions to a 96-well white, clear-bottom plate (Greiner Bio-One) at 100 µl per well, also including PBS/medium only controls.
- Add 100 µl luciferase substrate to each well.
- Read luminescence 3 times at 5 min intervals**.**

# 9 Species identification

The method used is based on that of Schönian et al., (2003) Diagn Microbiol Infect Dis; 47(1):349-358. It takes advantage of the presence of *Hae*III restriction fragment length polymorphisms in the ribosomal internal transcribed spacer locus (ITS1) in different *Leishmania* species. Although not comprehensive, this technique is useful for quick determination of the species but more detailed karyotype analysis would be necessary when dealing with a specific member of, for example, the *Leishmania* viannia sub-genus (*L. braziliensis*, *L. guyanensis* and *L. panamensis*). The same is true of the *L. donovani* sub-genus (*L. donovani, L. infantum* and *L. chagasi*).

## 9.1 PCR

Set up PCR reactions as follows – 1 PCR for each species:

| | |
|---|---|
| 5 µl | 10x PCR buffer |
| 1 µl | genomic DNA (standard Qiagen DNeasy genomic DNA prep) |
| 1.5 µl | OL1853 (CTGGATCATTTTCCGATG) |
| 1.5 µl | OL1854 (TGATACCACTTATCGCACTT) |
| 1 µl | Taq DNA polymerase |
| 40 µl | $dH_2O$ |

The PCR conditions to be used are as follows (if a tube sensor or sim-tube is used on your particular PCR machine then drop 10 seconds from each step in the following reaction):

$95^oC$, 40 s
$95^oC$, 40 s
$53^oC$, 40 s } x 35
$72^oC$, 50 s
$4^oC$ -> ∞

Purify each PCR product using Qiaquick columns according to manufacturer's instructions (Qiagen). Elute PCR products in 50 µl $dH_2O$.

## 9.2 Restriction digests

Set up restriction digests with the purified PCR products as follows:

| | |
|---|---|
| 43 µl | eluted PCR product |
| 5 µl | NEB Buffer 2 or CutSmart buffer |
| 2 µl | *Hae*III |

PCR products should be digested for 2 h at $37^oC$.
Digests are run out on a 2% LE Agarose + 1% regular agarose gel in 0.5x TBE.

The following patterns can be expected for the various species commonly used in this and other labs.

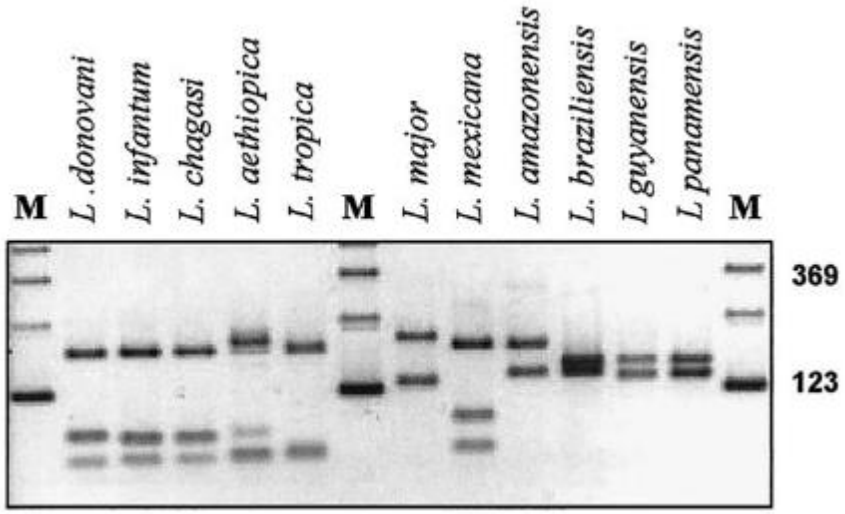



**Figure**. Taken from **Schönian et al., (2003) Diagn Microbiol Infect Dis; 47(1):349-358**

# Appendix A: Molecular analysis of *Leishmania* mutants

There are three methods that can be used to check that the cell lines generated are actually a knock-out, re-expressor or over-expressor:

1. PCR
2. Southern blot
3. Western blot

**IMPORTANT:** A Southern blot needs to be performed to check KO cell lines. A Western blot is also a good thing to do if you have an antibody. PCR is a quick way of screening potential KO lines but it has several problem attached to it (see below).

## A1 PCR

### *A1.1 Knock-out*

It is a quick and easy method but it can produce artefacts as you are amplifying DNA fragments (PCR is an exponential amplification process). Even a small amount of DNA from a non-transfected dead cell can produce a band.

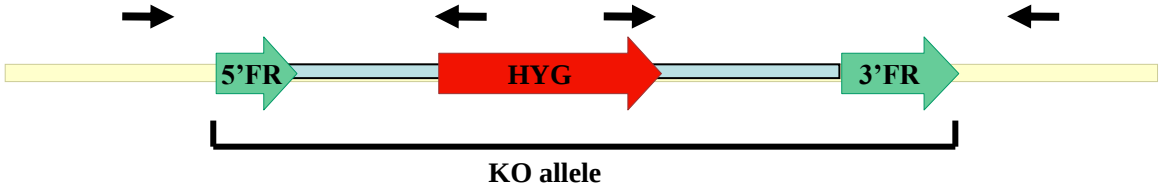



**Figure 1.** Diagram of a hygromycin resistance (HYG, indicated in red) KO allele. The black arrows represent primers. The 5' and 3' flanking regions (5' FR and 3' FR) used for the transfection are indicated in green.

To check the insertion of the DNA fragment (resistance cassette) in the right locus (the gene of interest) use a primer located outside the KO allele and a second primer ideally located in the resistance cassette. With two PCR reactions, you can check the correct insertion of the DNA fragment. Compare the size of the PCR fragments obtained with the predicted sizes, if they match you have correct integration.

**IMPORTANT:** A PCR will not tell you if the wild type (WT) gene is still present because of tetraploidy or reintegration somewhere else in the genome. The WT gene may still be amplified from a gDNA preparation following transfection even though the line is a true KO. This has been observed and is most likely due to the traces of gDNA of dead cell that did not transfect.

### *A1.2 pRIB (integration in ribosomal locus)*

Prior to integration, pRIB constructs containing a gene of interest will be digested with *Pac*I and *Pme*I to release the integration cassette, which should be run on a gel and purified. The integration cassette should be transfected and cells should be selected using appropriate antibiotics. Quick PCR tests can be carried out to determine if the pRIB construct has integrated into the ribosomal locus using isolated DNA from antibiotic-resistant cells.

Two tests should be done to confirm that the fragments have correctly integrated and not, as has been seen previously, remained as episomal copies.

1. OL2380 (CATTCCGTGCGAAAGCCGG) is a forward primer that anneals immediately upstream of all possible integration sites in the ribosomal locus. OL2380 should be used with a gene-specific reverse primer to show integration. Note that this primer has been shown by us to work in *L. major*, *L. infantum*, *L. braziliensis* and *L. mexicana* (unpublished observation).

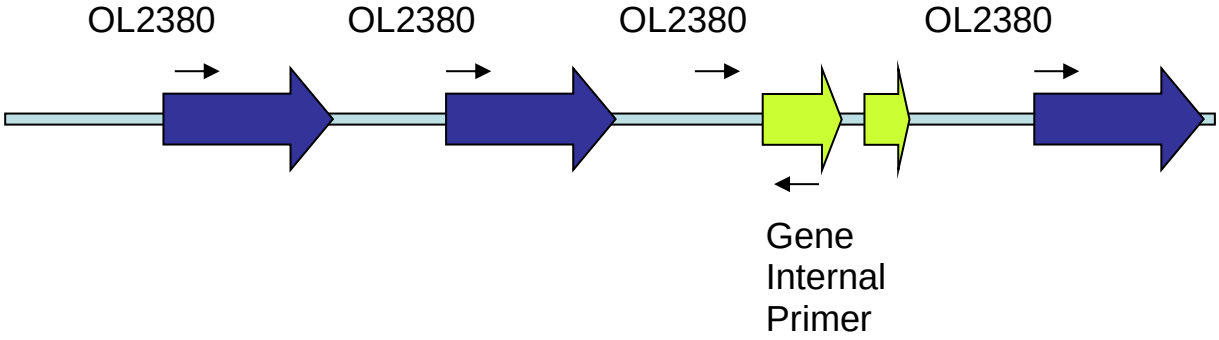



**Figure.** Annealing sites of the forward primer, OL2380, in the ribosomal locus. A gene-specific reverse primer will be needed for PCR in combination with OL2380 to show integration.

2. To confirm that there is no episome, OL556 (GCCTCTTCGCTATTACGC) should used together with a reverse primer internal to the gene being integrated. If this PCR is negative, while the PCR in step 1 is positive, then this probably indicates integration.

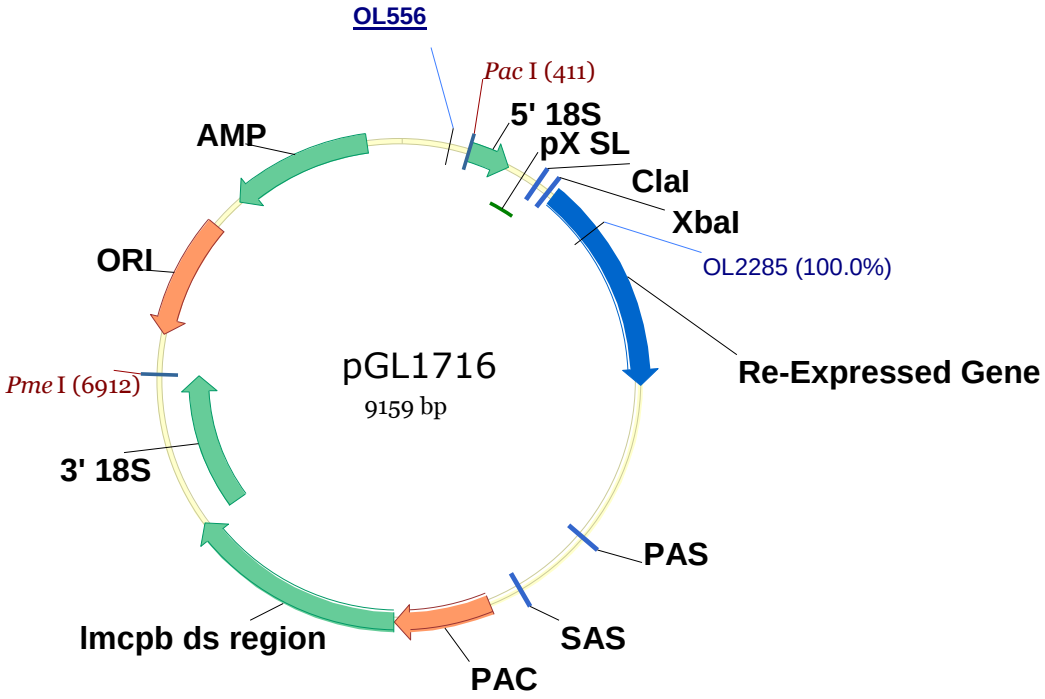



**Figure.** Example of a pRIB construct. The forward primer, OL556, anneals within the backbone of the pRIB construct. A gene-specific reverse primer used in combination with OL556 will show whether episomal copies are present or not.

Southern blot detection should then be used to confirm integration.

### *A1.3 pXG (over-expressors)*

Use OL320 and OL321. These primers are located on either side of the multiple cloning site of pXG. An empty pGL102 will generate a 190 bp PCR fragment. This PCR is not very useful. To see if the overexpressing vector is present, a Southern blot or Western blot are recommended.

## A2 Southern blot

Following a PCR screen of the potential KO cell lines, a Southern blot should be performed.

### *A2.1 DNA preparation*

Digest 5 µg of DNA for each line to be tested. Remember to include a WT control.

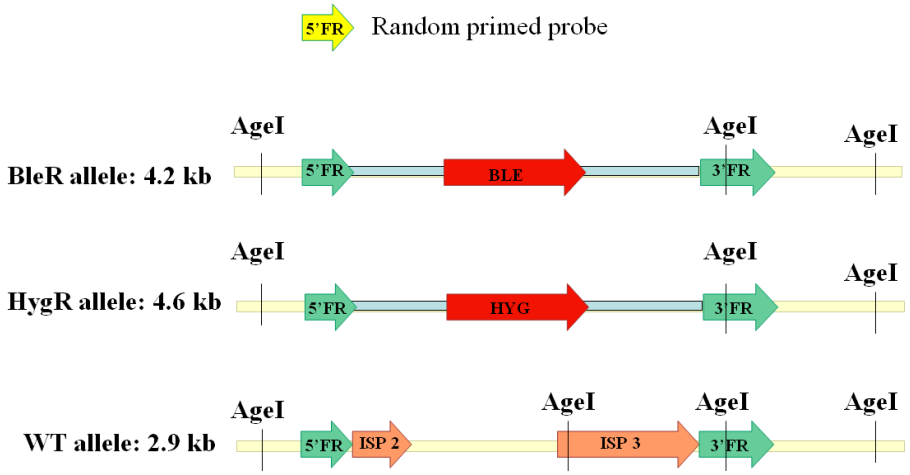



**Figure.** Example of AgeI digestion.

### *A2.2 Gel running and treatment*

Run digested DNA down a 0.8% agarose gel of sufficient length to separate the fragments and photograph beside a UV ruler so that fragment sizes can be calculated.

Depurination – Removes purines and fragments DNA making it easier to transfer – 10 min in 0.25M HCl.

Denature – 15-30 min in 1.5 M NaCl, 0.5M NaOH then rinse in $H_2O$.

Neutralise – 15-30 min in 3M NaCl, 0.5M Tris-HCl pH7.

### *A2.3 Transfer*

Fill a tray with 20x SSC (3M NaCl, 0.3M sodium citrate)
Pre-soak membrane and blotting paper, including wick in 20x SSC. The may need to be pre-soaked in $H_2O$, depending on the type.
Lay the wick over the plastic support
Place the gel on the wick.
Place the membrane on the gel and mark e.g. notch top right-hand corner, then cover with 2 layers of blotting paper cut to size, a thick wad of paper towels and a glass or Perspex plate to evenly spread the load of a weight.

Cover any exposed blotting paper with parafilm or cling film so that capillary action is focused on the gel.
Leave overnight and then carefully take apart keeping the membrane and gel together until wells are marked in pencil.
Wash membrane in 2x SSC for 10-20 min then place between 2 sheets of blotting paper and cross link the DNA.
Blot can now be kept indefinitely.

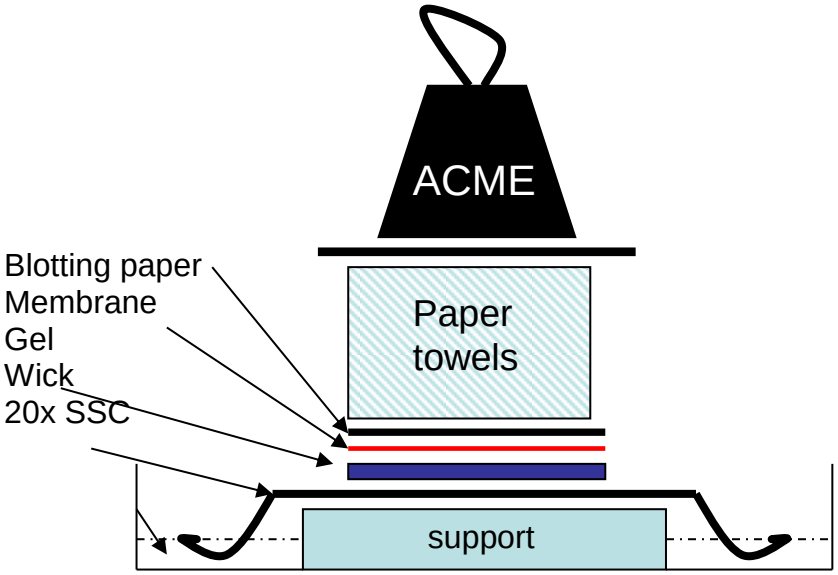



**Figure.** Diagram to show the setup for a Southern blot transfer.

### *A2.4 Probing blot*

Traditionally radio-labelled probes have been used in Southern blots (See Figure below); however, the non-radioactive labelling kits are now very sensitive and are often used instead. Good results have been achieved using the Amersham AlkPhos Direct labelling Kit and CPD-Star Detection Reagent. The kit is used in accordance with the manufacturer's instructions except that the recommended hybridisation temperature of 55$^{o}$C is generally too low for *Leishmania* which have a high GC content.

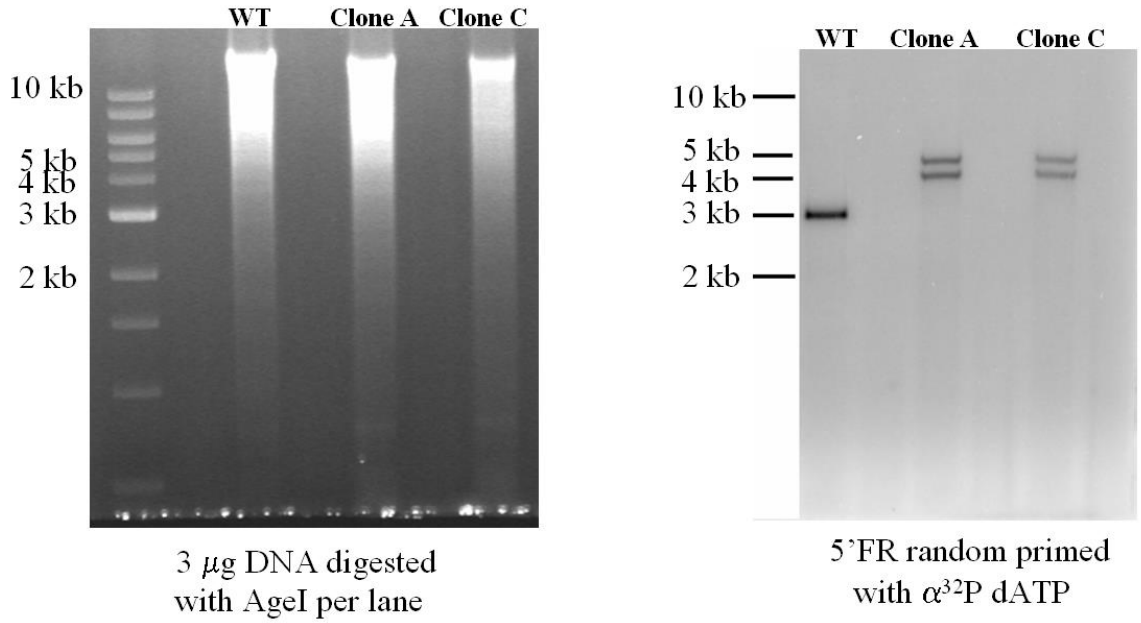
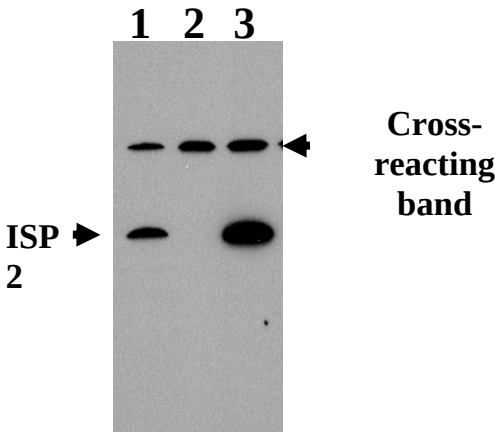



**Figure.** Southern blot showing the wild-type (WT) allele for *L. major ISP2* and *ISP3* and the 2 knock-out (KO) alleles (hygromycin resistance, HYG; bleomycin resistance, BLE). The restriction sites for *Age*I for each allele are shown. The sizes that one would expect to see on a Southern blot following an *Age*I digest and hybridisation with a radio-labelled 5' flanking region DNA probe are also shown. The DNA for each line, WT, KO clone A and KO clone C, (3 µg/sample) was digested with *Age*I ran on a 0.8% agarose gel, transferred on a nylon or nitrocellulose membrane, and hybridised with a radio-labelled 5' flanking region DNA probe. The radioactive bands on the Southern blot match the prediction and confirm that the WT allele has been inactivated. A western blot needs to be performed to show that the genes knocked-out are no longer expressed.

A Southern blot can also be performed to show whether a particular cell line is over-expressing (episome) or re-expressing (integrated) a specific gene. The choice of restriction enzyme(s) and probe will determine what you can detect.

## A3 Western blot

If the antibodies are available, a western blot can be used to check whether the protein is no longer expressed in the knock-out, or if it is re-expressed or over-expressed in respective cell lines. A second antibody is required as a loading control.
Run cell extracts ($10^7$ cells/lane, usually prepared at $10^7$ cells/10 µl) for each line to be investigated on a SDS-PAGE and transfer the protein onto a PVDF membrane.




**1- WT**
**2- KO (clone A)**
**3- KO + ISP2 overexpressor**

**Figure.** Western blot using purified antibodies against *L. major* ISP2. The cross-reacting band shows each lane is loaded with the same amount of etract. In lane one, wild type (WT) sample, ISP2 is visible. In lane 2, ISP2 knock-out (KO), no band is visible. In lane 3, an ISP2 over-expressing construct transfected into the KO strain and produces more ISP2 than the WT strain under 25 μg/ml neomycin selection.