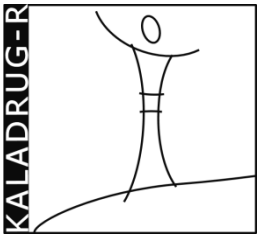
KALADRUG-R: Laboratory SOP#11



Culturing reference *L. donovani* strains

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Note: this protocol is designed for culturing strains already well adapted to *in vitro* conditions; another SOP (clinical SOP #A, G and H) is provided, describing culture conditions for primary isolation of parasites from bone marrow, spleen and blood (a bi-phasic medium is used for primary isolation). If a reference strain shows difficulties to grow with monophasic medium, it might be recommended to pass it again on bi-phasic medium until it is grows well again and then transfer to monophasic medium.

A. Materials needed

1. sterile monophasic medium suitable for *Leishmania donovani* culturing. Options include HOMEM (composition see annex I), M199 (recipe described in clinical protocol H)
2. sterile heat inactivated foetal serum (HIFCS)
3. sterile 75cm² culture flasks
4. sterile 50 mL universals for freezing -20°C
5. sterile pipette filtertips for microPipette 20-200µL
6. micro-pipette 20-200µL
7. sterile pipettes 5mL-10mL -25 mL & pipette pump
8. syringes 25 mL+ 21 ½ gauge needle
9. syringe filters 22 µm
10. 26°C incubator

B. Heat inactivation of foetal calf serum

1. Aliquot 500 ml foetal calf serum into > 40 ml (<50 ml)/50 ml universals
2. Make tubes water tight with para-film
3. Put rack in pre-warmed waterbath @ 56°C and include mock universal filled with equal volume of water and thermometer
4. As soon as thermometer in mock tube indicates 56°C, start timing 30 min

5. After inactivation, allow tubes to cool down and freeze at -20°C

C. Prepare culture medium

1. HIFCS 20% v/v (stored in 50 mL aliquots -20°C, thaw 1 night before use at 4°C, filter sterilise before use)
2. monophasic culture medium 80% v/v (stock stored at 4°C)
3. carefully mix before use

D. Start culture from stabilate

1. Take out vials from liquid nitrogen or -70°C and put them for 1 min at 37°C in a WWB containing dettol or another disinfectant. transfer to parasitology lab. Keep the vials on room temperature, transport them upright in a rack
2. Put the rack + vials in flow and spray it with 70% alcohol, leave the vials to thaw (don 't shake them, just leave them standing in the rack)
3. Prepare new labelled flask with 5 mL fresh monophasic medium + 20% HIFCS
4. Take the matching stabilate vial (double check identity), check if thawed without shaking it (try to keep the vial upright)
5. IF thawed, open the vial as follows: use a tissue soaked in 70% alcohol and carefully unscrew the cap of the vial
6. Use a plastic Pasteur pipette to transfer the content of the vial to the prepared culture flask at once in a sterile manner
7. Close the culture flask, and shake carefully to mix the stabilate mixture with the culture medium
8. Check with the inverted microscope if the parasites are in good condition
9. Transfer culture flask to 26°C incubator
10. Check culture regularly (every 48 hours) and if looking good (high density $>10^7$/ml, no contamination), passage 150 µL to a new flask with 5 mL HOMEM + 20% HIFCS
11. From this point the parasites can be cultured following the SOP below

E. Standard subpassage of *L. donovani* cultures:

1. Label new flask with strain name, date and new passage no. of strain as written in laboratory SOP#13
2. Add 5 ml fresh medium (monophasic medium + 20% HIFCS) to new flask
3. Inspect D3-4 stationary phase culture for good growth (generally > $1.5x10^7$ par/mL) and conditions

4. If culture is considered in good condition and to be in mid-late stationary phase, passage 100 µL of this culture to a new flask with 5 mL fresh medium (HOMEM+20% HIFCS) in a sterile manner.
5. Close cap of new culture, and shake carefully to mix parasite inoculum with fresh medium
6. The new culture can now be grown for 6-8 days (depends on strain) at 26°C, till mid-late stationary phase is achieved.

Annex I: Composition HOMEM MEDIUM

	For 1 Litre	For 10 Litres
S-MEM (Eagle)*	1 x 1 Litre pack	1 x 10 Litre pack
Glucose	1-2 g***	10 -20 g***
Sodium bicarbonate **	0.3 g	3.0 g
Sodium pyruvate	0.11 g	1.1 g
p-Aminobenzoic acid	1 mg	10 mg
Biotin	0.1 mg	1 mg
HEPES	4.77 – 5.96 g***	47.7 – 59.6 g***
MEM amino acids	10 mL	100 mL
MEM Non-essential amino acids	10 mL	100 mL

*Obtained from Gibco. Cat. No.:-1140-082

**The original formula called for 2.2 g $NaHCO_3$ per Litre, but using HEPES buffer, this amount is far too high and the amount of $NaHCO_3$ used has been gradually lowered. It has been found that 0.3 g/L is quite sufficient.

***We normally use the larger amount.

Make up to pH 7.5 – 7.6 as the pH will, after sterilizing drop to pH 7.0 – 7.2.

The original formula for HOMEM called for the addition of Haemin and Folic acid after sterilization of the other compounds, but this has been found to be unnecessary with the particular strain of *L.m.mexicana* used here (Probably enough in the serum).

Filter sterilize using 0.22µm Millipore filter into sterile bottles and store at 4°C. Mark the first and last bottles and incubate at 37°C as a check for contamination.

For longer storage, freeze at -20°C. Medium appears to be stable at -20°C for at least 6 months and possibly longer.