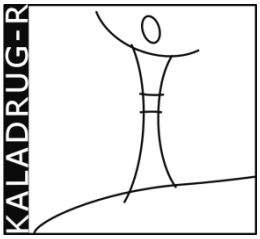
KALADRUG-R: Laboratory SOP #9

Miltefosine susceptibility testing *L. donovani* intracellular amastigotes



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A. Material required

1. x 10 mL syringes (x=no. of mice)
2. x 21 ½ gauge needles (green)
3. x 26 3/8 gauge needles (brown)
4. 50 mL universals RT
5. 50 mL universals on ice
6. Forceps and sciccors
7. 7 mL and 15 ml Sterilin tubes for drug solutions
8. filter syringes 22µM, suitable for small volumes
9. cavity slides (Lab Tek Chamber Slide System 16-well Nunc Cat. No. 178599, VWR)
10. Crystalline Miltefosine (Paladin)
11. 2% starch solution

B. Solutions required

1. RPMI 1640 (+ L-glutamine, + 25 mM HEPES) (PAA, Catalog no. E15-842)(=Basic RPMI)
2. Gentamycin 10 mg/mL (Sigma, Cat. No. G1272)
3. penicillin/streptomycin (100x) PAA laboratories Cat. No. P11-010 (contents: 10.000 u Pen/ 10mg/mL Strep)
4. RPMI 1640 + HIFCS 10%v/v + 1%v/v Pen/Strep + 0.5% Gentamycin v/v(=Full RMPI)
5. Phosphate Buffer Saline (PBS)

C. Preparation of Miltefosine solutions

!!! Avoid direct contact with substance, irritating to eyes, toxicological properties have not been fully determined!!!!

1. Prepared stock solution MIL 3 mg/mL by weighing 0.030 g MIL and dissolving in 10 mL PBS. The resulting solution should be completely transparent.
2. Filter this solution over 0.22 µM syringe filter for small volumes= **STOCK SOLUTION A (MIL 3000 µg/mL)**
3. Aliquot the stock solution in volumes of 1 mL and store these aliquots @ -20°C for max. 3 months
4. When starting a susceptibility assay, thaw 1 stock solution A & remember to manipulate sterile
5. Add 300 µL stock A to 600 µL sterile PBS = **stock solution B (MIL 1000 µg/mL)**
6. Add 100 µL stock A to 900 µL sterile PBS = **stock solution C (MIL 300 µg/mL)**
7. Add 100 µL stock C to 900 µL sterile PBS = **stock solution D (MIL 30 µg/mL)**
8. The stock solutions B, C, D should be FRESHLY diluted 1/100 in FULL RPMI for drugging *Leishmania* infected macrophages resulting in final concentrations of
    - solution B = 10 µg/mL
    - solution C = 3 µg/mL
    - solution D = 0.3 µg/mL

D. Extraction and plating murine peritoneal macrophages

1. Inject 2% starch solution in peritoneum of mice to stimulate macrophage production 48hrs before harvest
2. Prepare sufficient (dependent on no. mouse) basic RPMI 1640 with 1% Gentamycin (50 mL basic RPMI + 0.5 mL Gentamycin) in Falcon tube
3. Distribute prepared medium in syringes, 10 mL/ syringe for normal mice, 6 mL/ syringe for BALB/C mice
4. Collect the mice, spread the mouse on their backs and spray belly with 70% ethanol.
5. Snip mouse where the belly button would be (V. small nick!), and pull off the skin (first pull apart, and then sideways = deglove)
6. Spray the inside with 70% ethanol
7. Put a 21 ½ gauge needle onto a charged syringe
8. Using forceps, pull up the membrane at the sternum and insert the needle (just under membrane, bevel facing upwards)
9. Gently fill the cavity with the RPMI (membrane should be thaught)
10. Hold the mouse by base of tail and shoulders and wiggle for 20 secs (allowing the RPMI to rinse the cavity)
11. Face the mouse towards you on its back and insert a 26 3/8 gauge (brown) needle (with the empty syringe) into the mouse's side (bevel facing body
12. Pull the skin taught against the needle and remove the fluid from the cavity, change sides when one side has been depleted
13. When all fluid has been extracted (6-8 mL), transfer the fluid into a 50 mL Falcon on ice.

14. Spin down the collected macrophages @ 1700 rpm for 10 min @ 4°C (a reddish pellet should result, red indicates the presence of RBC)
15. Remove the SN and add 10 mL full RPMI
16. Resuspend the pellet with plastic pasteur pipette and take aliquot to count (macrophages are slightly larger than RBC's and more granular looking, without characteristic shape of RBC)
17. Adjust concentration to 5 x 10^5 cells/mL using full RPMI.
18. Put 10 mL 100 µL/well for cavity, prepare 3 separate cavity slides, one for check @ 24hrs (4 wells/strain), one for untreated control infection 5 days (4 wells/strain) & 1 for treated infection 5 days(4 wells/conc. drug/strain)
19. Incubate @ 37°C, 5% CO_2 for 24 hrs (cells will adhere to surface, not multiply!)

Note: The macrophages can be kept for up to a week after plating if necessary, but one must keep the media covering them fresh and suitably high. They are best used immediately after extraction.

E. *In vitro* Miltefosine susceptibility testing of *L. donovani* in peritoneal macrophages

Control strain to be included for all susceptibility tests:
For *L. donovani* Nepal: BPK282/0 clone 4 or BPK206/0 clone 10
For *L. donovani* India: AG83

1. Count late stationary promastigotes in culture
2. Dilute to 5 x10^6 / mL for infection at 1 mf:10 parasite ratio using full RPMI
3. Add add 100 µL parasite suspension of 5 x10^6 / mL in each well of the cavity slides.
4. Put infected cultures at 37°C, 5% CO_2
5. @ 24hrs:

- For controls slide 24 hrs:
  - Remove medium with pastette
  - Wash with basic RPMI 1640 up to 3 times (fill with pastette)
  - Remove the wells from the slide
  - Methanol fix for about 1 min.
  - Shake off the methanol
  - Remove the plastic rings of the slide
  - Giemsa stain (1:10 dilution) for 10 min
    (use 10 mL syringe, 4.5 mL H_20 + 0.5 mL Giemsa, and drip out over slide)
  - Rinse off Giemsa stain with water
  - Count 100 macrophages and determine % infection
- For other slides:
  - Prepare 1/100 dilutions of the 4 stocks of drug-solutions in full RPMI (see point C)
  - Cover with 200 µL of WORKSOLUTION drug, 4 wells for each concentration

- Put back at 37°C, 5% CO_2

6. @ 5 days: stop infection and check cavity slides as described in step 5

F. Analysis results

1. Microscopically evaluate infection levels of all control wells and all treated wells: count % infected macrophages and average no. of amastigotes/macrophage
2. Determine infection index = no. of amastigotes/mf * % infected mf
3. Determine % survival ± stdev at each drug concentration by infection index in treated wells with infection index of untreated wells
4. Use sigmoid regression analysis to determine corresponding ED50 ± 95% confidence intervals SPSS, XLfit, Graphpad prism are software packages that can do this type of non-linear regression analysis.