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Screening of drugs against Leishmania amazonensis axenic amastigotes *in vitro*

Protocol for culturing promastigotes of L. amazonensis (wild type) and differentiation in axenic amastigotes (Vermelho, et al; 2010)

PBHIL Medium (1L):
2g/L glucose
2g/L Peptone
2g/L Brain Heart Broth
0.25g/L Liver Infusion Broth
0.4g/L Sodium chloride
4g/L Potassium chloride
11.5g/L Monobasic sodium phosphate
3g/L Sodium hydroxide
0.01g/L Hemin; 10% FBS.

Add all the ingrediens to 1L of MiliQ water.
Separate in 2 beckers of 500ml each.
Adjust the pH to 7.2 (promastigotes) and 4.6 (axenic amastigotes).
Add 50ml of FBS to each of the beckers and filter.

1) Promastigotes culture – Grow in PBHIL medium – pH 7.2 at 26 ºC. Medium can be changed once a week.
2) Axenic Amastigotes – Spind down promastigotes from stationary phase (5 to 7 days) at 3000rpm for 10 min, break 1. Discard all the supernatant and ressuspend in PBHIL ph 4.6 for counting. Dilute the parasites at $5x10^6$ parasites/ml and incubate them at 32-34ºC for 5-7 days. Differentiation should be followed by looking in the microscope.

Protocol for inhibition assay on *L. amazonensis* axenic amastigotes (wild type)

The assay is performed in 96 well transparent sterile plates for 96 hours. Each compound should be tested in duplicate. The controls in triplicate.

Reagents:

PBHIL medium (pH 4.6)
Alamar Blue (Sigma)

Controls:

Medium alone, parasites alone, parasites + 100uM of Ionomycin

Protocol:

- Thaw out compounds
- Spin parasites for 10 min at 3000 rpm (9 acceleration, 1 break).
- In the meanwhile, add 100uL of PBHIL medium (pH.4.6) per well in the 96 well plate
- Vortex compounds
- Add 2uL of each compound + 98uL of fresh medium in the first row, pipette up and down to mix and then transfer 100uL to the next row in order to make serial dilutions
- After centrifugation, discard medium by aspiration carefully
- Resuspend parasites in warm medium and count them in Newbauer chamber
- Dilute parasites at 10^7 cells/ml (10^6 cells/well) and plate 100uL per well with multichannel pipette
- Incubate at 32°C for 96h.
- Add 40uL of Alamar blue per well
- Incubate at 32°C for 5 hours
- Read fluorescence at excitation 530nm and 590nm emission wavelength

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