



## **Screening of drugs against *Leishmania amazonensis* infection of macrophages *in vitro***

### **Protocol for culturing promastigotes of *L. amazonensis* (expressing beta-lactamase from Fred Buckner\*) and immortalized macrophages (J774)**

Both, parasites and macrophages should be cultured in RPMI supplemented with 10% of FBS and 1% PSG. Both can be frozen by adding medium with 10% of glyceraldeyde and placing the vials at -80°C for 24h and then transferring to liquid nitrogen.

*L. amazonensis* – Promastigotes forms grow at 26°C and medium can be changed once a week.

J774 – Macrophages grow at 37°C with 5% of CO<sub>2</sub>. Discard the medium and wash the flask once with PBS 1X sterile. Add Trypsin enough to cover the surface and place the flask inside of incubator for a couple of minutes. Check in the microscope to check when the majority of cells are detached and add fresh medium to stop the reaction. Spin down at 1200rpm for 5 min. Discard the old medium and add fresh medium for counting. Plate the cells at the desired concentration in a new cell culture flask.

### **Protocol for inhibition assay on intramacrophage amastigotes of *L. amazonensis* expressing Beta-lactamase**

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:

**RPMI** medium supplemented with 10% FBS and 1% PSG.

**CENTA™ β-Lactamase Substrate** (EMD Chemicals - cat number 219475)

**Nonidet P-40** (Igepal CA 360, Fluka, cat number 56741)

Controls:

Medium alone, Macrophages alone, Macrophages + *L. amazonensis* and Macrophages + *L. amazonensis* + 100uM of Ionomycin

Protocol:

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- Trypsinize the macrophages (J774) and plate them at the concentration  $5 \times 10^4$ / well ( $5 \times 10^5$ /ml) in 100uL.
- Incubate for 4 hours at 37°C, 5% CO<sub>2</sub>, for the macrophages to attach
- Spin down *L. amazonensis* in stationary phase (5-7 days) at 3000 rpm for 8 min (9 acceleration, 1 break)
- Dilute them at  $125 \times 10^5$  parasites/ml (25 parasites : 1 macrophage) and add 100uL to attached macropages.
- Incubate overnight at 32°C, 5% CO<sub>2</sub>.
- In the next day, remove the medium by aspiration and add 200uL of warm PBS per well (very careful) for removing the parasites that didn't infect.
- Aspirate the PBS and add 100uL of fresh medium.
- 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. (Pipette carefully to not deattach the macrophages.
- Add more 100uL of fresh medium
- Incubate for 96 h at 32°C, 5% CO<sub>2</sub>.
- After 96h, prepare the substrate solution: 0.1% Nonidet P-40 + 100uM of CENTA
- Remove the media and add 100uL of the substrate per well.
- Incubate for 4 hours at 32°C.
- Read the absorbance at 405nm.

\***Buckner FS, Wilson AJ.** Colorimetric assay for screening compounds against Leishmania amastigotes grown in macrophages. **Am J Trop Med Hyg** 2005 May;**72(5):600-5.**

November 1, 2016