

## **Subculture and cryopreservation of *Leishmania donovani***

From Ainslie Lab @ UNC [ainslielab.web.unc.edu](http://ainslielab.web.unc.edu)

### ***Leishmania donovani* (ATCC 30030)**

#### **Composition of modified M199 medium (500mL):**

- 1) M199 medium (Sigma-M4530) 444 mL
- 2) Heat inactivated FBS 50 mL (10%)
- 3) Hemin soln (5 mg/mL) 1 mL
- 4) Pen-Strep 5 mL (1%, 100 IU/mL pen and 100 µg/mL strep)

#### **Composition of hemin solution (5 mL):**

- 1) Triethanolamine (Sigma-90279) 2.5 mL
- 2) Hemin (Sigma-H9039) 25 mg
- 3) Distilled water 2.5 mL

#### **Equipment:**

- 1) 37°C water bath
- 2) Centrifuge machine
- 3) 25°C incubator
- 4) Phase contrast microscope
- 5) Hemocytometer

#### **Thawing frozen ampoules and culture initiation:**

- 1) Take ampoule from liquid nitrogen.
- 2) To thaw a frozen ampoule, place in a 37°C water bath until thawed (2-3 mins). Immerse the ampoule just sufficient to cover the frozen material. Do not agitate the ampoule.
- 3) Transfer the content aseptically (in the class 2 cabinet) in 10 mL of modified M199 medium
- 4) Spin down @ 1500 g, 10 mins and aspirate supernatant.
- 5) Dissolve pellet in 500 µL of modified M199 medium, and subsequently transfer to a T25 flask containing 9.5 mL of fresh modified M199 medium (alternatively, can also be cultured in a screw-capped borosilicate test tube containing 9.5 mL of fresh modified M199 medium with the cap screwed on tightly. But, very slower growth rate observed when cultured in test tube compared to T25 flask)
- 6) Put in a 25°C incubator and monitor the culture day by day in a phase contrast microscope.
- 7) Amastigotes should turn to promastigotes in 3-5 days.

#### **Subculturing / Passage of the promastigotes:**

- 1) For subculturing the *Leishmania* promastigotes need to be passage every 2 to 3 days (Usually Monday, Wednesday and Friday of the week). If subculturing in a screw-capped borosilicate test tube then twice in a week (e.g. Tuesday and Friday of the week).
- 2) For subculturing/serial passage take 1 mL from the existing culture (resuspend the culture with a pipette to dissociate clumps) and put in to 9 mL of fresh complete M199 medium.
- 3) Keep the number of Passage under 15 as promastigotes may lose their infectivity during serial passaging. This is especially important if you want to use the cells for in vivo infection.

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### **Counting *Leishmania* by Hemocytometer**

#### **Equipment:**

- 1) Microscope
- 2) Hemocytometer
- 3) 1.5 mL Eppendorf tube
- 4) Cell counter

#### **Reagents:**

2% formaldehyde (10% neutral buffered formalin diluted to 5% with PBS)

### **Cleaning Hemocytometer**

- 1) Clean the hemocytometer with ethanol and Kim wipe tissues
- 2) Put the cover slip on the specific place of hemocytometer.

### **Preparation of *L. donovani* for counting**

- 1) Gently mix the culture of the T25 flask by using a 5/10 mL serological pipette to ensure the cells are evenly distributed.
- 2) Take 50  $\mu$ L of cells and place a 1.5 mL Eppendorf tube containing 50  $\mu$ L of 2% formaldehyde and mix thoroughly
- 3) Place 10  $\mu$ L of fixed cells on the Neubauer hemocytometer and leave at RT for 5 mins to allow the cells to settle down.
- 4) Place the hemocytometer on a microscope. Focus on the gridlines of the hemocytometer preferably with a 10X objective.
- 5) Using a hand tally counter, count the cells in one of the 16-squares. Cells counted as in they are within a square or on the top or right boundary line. Cells on the bottom or on the left boundary will not be counted.
- 6) Move the hemocytometer focus to the next 16-squares (corner) and carry on counting until all 4 sets of 16-squares are counted.

### **Calculation of *Leishmania* number**

Average *Leishmania* count of four of big 16-squares \* dilution factor \*  $10^4$  Ld/mL

### **Cryopreservation / making stock:**

- 1) Gently mix the culture of the T25 flask by using a 5/10 mL serological pipette and harvest the cells by centrifuging 1500 g for 10 mins.
- 2) Suspend the pellet with fresh modified M199 medium and count the no of *Leishmania donovani* in a haemocytometer.
- 3) Adjust the cell concentration @  $2 \times 10^7$  Ld/mL with fresh modified M199 medium.
- 4) While cells were centrifuging prepare a 10% (v/v) sterile DMSO in fresh modified M199 medium. The DMSO solution when first prepared will warm up due to chemical heat. The solution should be allowed to return to room temperature prior to use.
- 5) Mix the cell suspension (from step 3) and the DMSO solution in equal portion. The final concentration of the *Leishmania* will be  $1 \times 10^7$ /mL and 5% (v/v) DMSO. The time from the mixing of the cell preparation and DMSO stock solution before the freezing process begun should be no more than 15 mins.
- 6) Dispense in 1 mL aliquots into 2-mL sterile plastic screw-capped cryovial.
- 7) Place the ampoules in a Nalgene 1C freezing apparatus. Place the apparatus at  $-80^\circ\text{C}$  for 2-3h and then plunge the ampoules into liquid nitrogen.
- 8) Store either in vapor or liquid phase of a nitrogen chamber.