SOP: SP060

Preparation of Infectivity stocks

Materials and Reagents:

1. PB media (See SOP M020)
2. *Mycobacterium tuberculosis* (strain of interest), frozen stock
3. Biosafety cabinet (BSC)
4. Electric pipette
5. 1 mL, 10 mL, 25 mL sterile pipets
6. Pipet boat with Amphyl (or equivalent anti-tubercular disinfectant)
7. 25 mL glass tubes with screw caps, rack
8. Sterile plastic loops, individually wrapped
9. Nutrient agar plates
10. 50 mL falcon tubes
11. 37 ºC warm room
12. 250 mL individually wrapped, sterile plastic corning flask
13. Glycerol, sterile
14. 21 gauge needle (or larger)
15. 20 mL syringe
16. Cryovials
17. 7H9 + OADC containing 0.1% Tween-80
18. 20% Tween-80
19. 7H11 plates (4 per sample)

Preparation of Seed Stocks:

1._____ Prepare an autoclaved, sterile glass with tube 9 mL of PB media.

2._____ Passage one: From one stock vial of *Mtb* (Note 1), transfer 1 mL of *Mtb* to glass tube.

3._____ Incubate the tube at 37 ºC without agitation for a few weeks, until pellicle has formed (Note 2).

4._____ Passage two: Once pellicle is confluent, it must be passaged (up-scaled). Remove culture from the warm room carefully, as to not disturb the pellicle.

5._____ Prepare sterile 50 mL falcon tube with 25 mL of PB media.

6._____ Using a plastic loop, carefully transfer the pellicle from the glass tube into the Falcon tube.

7._____ Incubate the tube at 37 ºC without agitation for a few weeks, until a confluent pellicle has formed. Remove culture from the warm room carefully, as to not disturb the pellicle.

8._____ Passage three: Prepare sterile 250 mL corning flask with 100 mL of PB media.

9._____ Using a plastic loop, carefully transfer the pellicle from the Falcon tube into the flask.

10.____ Incubate the tube at 37 ºC without agitation for a few weeks, until a confluent pellicle has formed. Remove culture from the warm room carefully, as to not disturb the pellicle.

Bottling of Seed Stocks:

1._____ Prepare sterile 50 mL Falcon tube with 25 mL of PB media with 20% glycerol.

2._____ When the pellicle is confluent, transfer to the 50 mL Falcon tube.

3._____ Use a 20 mL syringe with a 21 gauge needle to carefully pipette bacteria up and down to break up clumps (Note 3).

4._____ After allowing the sample to settle out (10-15 min), remove the media containing the resuspended bacteria (be sure to avoid any bacterial clumps which have settled out) and add it to a new 50 mL falcon tube.

5._____ Bath sonicate the suspension 3 * 10 sec each

6._____ Transfer resuspened organisms (once again, avoiding clumps) – as 1 mL aliquots, into cryovials.

7._____ Label the vials as seed stock with the strain name/number, your initials, date.

8._____ Store stocks at -80.

Growth of Working Stocks of *Mtb* from Seed Stocks:

1._____ Prepare an autoclaved, sterile glass tube with 9 mL of 7H9+OADC medium containing 0.1% Tween-80.

2._____ Passage one: From one vial of seed stock, transfer 1 mL to the prepared glass tube.

3._____ Incubate the tube at 37 ^{o}C on a shaker.

4._____ Check the culture daily by shaking the tube (look for a white swirl). After shaking, a frothy layer is noticeable above the liquid – this is the tween (Note 4).

5._____ Over time, the culture will turn a milky white color – this indicates growth.

6._____ Passage two (at approximately 10 – 14 days - when culture is milky white): Prepare a 250 mL flask with 50 mL of 7H9+OADC medium containing 0.1% Tween-80.

7._____ Transfer 5 mL of culture into prepared 50 mL falcon tube.

8._____ Incubate the tube at 37 ^{o}C on a shaker (until milky white, as before).

Bottling of Working Stocks:

1._____ On the morning of the harvest, add 0.5 mL of sterile 20% Tween-80.

2._____ Incubate at 37 ^{o}C shaking (about 30 min).

3._____ During incubation, label vials with organism name/number, your initials, date.

4._____ Bath sonicate 3 * 10 sec

5._____ Using a sterile pipette, transfer 1 mL of culture to the vials (be sure to keep your bacteria in suspension by swirling the flask between transfers).

6._____ Store at -20 ^{o}C for at least 4 hours before transferring to the -80 ^{o}C freezer.

7._____ Two to three days later, take 2 vials and plate 100 ul for 10-fold dilutions of 10^5 – 10^8 onto 7H11 agar to determine CFU/mL for working stock. The concentration should be between 7 * 10^7 and 2 *10^8 CFU/mL.

Notes:

1. To check for contamination, streak one plate with organism from the vial prior to adding it to the tube.
2. The pellicle is a layer of organisms that grows on top of the liquid media. The remaining liquid below the pellicle should be clear with a little bit of growth on the bottom. It should be a little stringy from the pellicle, but not excessive or cloudy.

3. To avoid the use of needles, the bacterial clumps can be broken down with a 1 mL seriological pipet. After cultures settled and remove all but a couple mLs of media – pipet with a P200. Continue on to step 4.
4. As *Mtb* grows, the amount of tween is reduced and it may be necessary to add new tween. To do this, add 1 mL of 20% Tween-80 in 1 L of PB