SOP: PP001.3
Modified: 7-31-23 AS

Establishment of frozen stocks of *Mycobacterium tuberculosis*, small scale

Materials and Reagents:

1. *M. tuberculosis*, 1 mL frozen stock or growing culture (note 1)
2. Biosafety cabinet
3. 7H11 + OADC agar plate (note 2)
4. Inoculation loop, 10 µL
5. P-200 pipettor
6. P-200 tips, sterile, aerosol-resistant tips
7. Ziploc bag
8. 7H9+OADC+0.05% tween, 100 mL (note 4)
9. Disposable Corning Erlenmeyer flask, 250 mL, sterile
10. Cell scraper, sterile
11. Bunsen burner
12. Orbital platform shaker
13. Pipet, 1 mL, sterile
14. Serological pipettor
15. Sterile glass tube with cap (16 x 100 mm)
16. Spectrophotometer, visible light
17. Pipet boat, containing organism specific disinfectant
18. Sterile Falcon centrifuge tube (50 mL)
19. 100% glycerol stock solution, sterile
20. Pipet, 10 mL, sterile
21. Cryovials, 1.7 mL, sterile
22. Labels for cryovials
23. Cryovial box, 81-place
24. Ultra-Low Freezer, -80°C
25. Autoclave
26. Nutrient agar plates (note 8)
27. Safety glasses and other PPE (personal protective equipment)

Protocol:

1._____ Set up the biosafety cabinet inside the BLS-3 (SP0041b). Thaw a 1 mL frozen stock of *M. tuberculosis* or obtain a growing culture of *M. tuberculosis* (note 1).

2._____ Streak a small 7H11 agar plate with bacteria and spread to grow as a lawn (note 2).

3._____ Place inoculated plates in a Ziploc bag, seal, and incubate at 37ºC until a lawn has formed at ~4weeks (note 3).

4._____ Inside the biosafety cabinet, use a sterile cell scraper and aseptically transfer bacterial lawn from one small plate or half a large plate to 5 or 10 mL 7H9/OADC/0.05% tween or GAS/tween media in a glass tube containing a stir bar (note 4 and 5). Use more glass tubes for more aliquots.

5._____ Incubate in the warm room on a stir plate for 4-6 days (note 6).

6._____ Add 20% total volume of 100% sterile glycerol to media and mix well. Using a sterile 10 ml pipet, aseptically add 1 ml of cell suspension to each sterile 1.7 mL cryovial. Discard the pipet as before when finished (note 7). Measure and take note of OD. Culture should be very thick (OD600 1-2.5).

7._____

8._____ Place vials in a cryovial box, and snap-freeze at –80ºC.

9._____ Autoclave glassware, trash, and pipet boat, etc. according to BSL-3 Procedure.

10._____ Test vial for contamination by streaking stock on a small nutrient agar plate and grow as a lawn (note 8).

11._____ Seal plate in a Ziploc bag and incubate at 37°C. Check for rapid growth of a possible contaminant. If no contaminant is present after 2 weeks, the stocks are ready to be included in the inventory.

Notes:

1. A seed stock is often the oldest batch of stocks derived from an original source (history may be difficult to track on older strains). When possible, seed stocks are made from one colony of an original source streaked for isolation. Colony is then streaked and grown till confluent. Working stocks are derived from seed stocks. All virulent *M. tuberculosis* must be handled inside a BSL-3 facility.
2. Use a plate prepared according to SOP M009. Use a sterile cell scraper to transfer bacteria from a plate or slant; use an aerosol resistant tip and pipettor to transfer cells from a liquid culture (200 µL from frozen stock).
3. Depending upon the strain and amount of viable cells inoculated, a lawn could take 2 to 6 weeks to form.
4. 7H9+OADC+0.05% tween is prepared according SOP M006. GAS+tween is prepared according to SOP M002.
5. **Alternate Protocol A:**

   This protocol works well when larger volumes are needed

   - Scrape cells and aseptically transfer into 50 mL 7H9/OADC/0.05% tween or GAS/tween media in a 250 mL Corning Erlenmeyer flask containing a sterile stir bar.
   - These can be incubated with stirring, or on an orbital platform rotating at approximately 65 RPM (enough to agitate the media so the cell clumps will disperse and the bacteria will be aerated). Better dispersal of cell clumps is achieved using a stir plate and sterile stir bar. Harvest is dependent on strain, but is typically 1-2 weeks.
   - Continue with SOP starting at step 6.

   Alternate Protocol B:

   This protocol results in very dilute stocks. Use with caution

   - Scrape cells and aseptically transfer to 100 mL of 7H9+OADC+ 0.05% tween in a 250 mL Corning Erlenmeyer flask containing a sterile stir bar.
   - These can be incubated with stirring, or on an orbital platform rotating at approximately 65 RPM (enough to agitate the media so the cell clumps will disperse and the bacteria will be aerated). Better dispersal of cell clumps is achieved using a stir plate and sterile stir bar. Harvest is dependent on strain, but typically 1-2 weeks.
   - When ready to harvest, aseptically add 12.5 mL of sterile 80% glycerol stock solution into a sterile 50 mL Falcon centrifuge tube.
   - Aseptically add 37.5 mL of suspended cells into the Falcon tube containing the glycerol stock solution and cap the tube.
   - Mix thoroughly by inverting several times (ensuring that the cap is properly sealed and tightened) or by pipetting.
   - Continue with SOP starting at step 6.
6. Labels containing strain, lot number, date, medium, and technician name should be made and placed on the vials.
7. A 5 mL volume will be ready to harvest sooner than a 10 mL volume.
8. Use plates prepared according to SOP M011.