

**SOP: PP001****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, small (10 x 150 mm) (note 2)
4. Inoculation loop, 10  $\mu$ l (note 2)
5. P-1000 pipettor (note 2)
6. P-1000 tips, sterile, aerosol-resistant tips (note 2)
7. Cotton swab, sterile (note 2)
8. Ziploc bags
9. GAS medium + 0.05% tween, 100 ml (note 4)
10. Disposable Corning Erlenmeyer flask, 250ml, sterile
11. Cell scraper, sterile
12. Bunsen burner
13. Orbital platform shaker
14. Pipet, 5 ml, sterile
15. Electric pipettor
16. Sterile glass tube with cap (16 x 100 mm)
17. Spectrophotometer, visible light
18. Pipet boat, containing 10% Lysol I.C. solution
19. Sterile Falcon centrifuge tube (50 ml)
20. 80 % glycerol stock solution, sterile
21. Pipet, 10ml, sterile
22. Cryovials, 1.7 ml, sterile (100) (note 7)
23. Cryostorage box, 100-place
24. Freezer, -80°C
25. Autoclave
26. Nutrient agar plates, (10 x 150mm) (note 8)

**Protocol:**

1. \_\_\_\_ Thaw a 1 ml frozen stock of *M. tuberculosis* or obtain a growing culture of *M. tuberculosis* (note 1).
2. \_\_\_\_ Streak a small 7H11 + OADC agar plate with bacteria and spread to grow as a lawn (note 2).
3. \_\_\_\_ Incubate at 37°C until a lawn has formed (note 3).
4. \_\_\_\_ Inside the biosafety cabinet, use a sterile cell scraper and aseptically transfer half of lawn to 100 ml of GAS + 0.05% tween in a 250 ml Corning Erlenmeyer flask (note 4).
5. \_\_\_\_ Gently flame the mouth of the Erlenmeyer flask and aseptically replace the lid.
6. \_\_\_\_ Incubate on an orbital platform shaker for 2 weeks at 37°C (note 5).
7. \_\_\_\_ Transfer the culture from the warm room into the biosafety cabinet.
8. \_\_\_\_ Using a sterile 5 ml pipet and an electric pipettor, transfer 5 ml of suspended cells to a sterile 16 x 100 mm glass tube and measure the O.D. A600; record in stock notebook. Discard the pipet into a pipet boat filled with 10% Lysol I. C. solution.
9. \_\_\_\_ Aseptically add 12.5 ml of sterile 80% glycerol stock solution into a sterile 50 ml Falcon centrifuge tube.

10. \_\_\_\_ Aseptically add 37.5 ml of suspended cells into the Falcon tube containing the glycerol stock solution and cap the tube.
11. \_\_\_\_ Mix thoroughly by inverting several times (note 6).
12. \_\_\_\_ 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).
13. \_\_\_\_ Cap the cryovials, place in a cryostorage box, and snap-freeze at  $-80^{\circ}\text{C}$ .
14. \_\_\_\_ Autoclave spent media, 16 x 100 mm tube with cells, trash, and pipet boat.
15. \_\_\_\_ After 1 or 2 weeks, thaw several stocks.
16. \_\_\_\_ Streak stocks individually on a small nutrient agar plates and grow as a lawn (note 8).
17. \_\_\_\_ Incubate at  $37^{\circ}\text{C}$  and 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 (note 9).

**Notes:**

1. Virulent *M. tuberculosis* must be handled inside a BSL-3 facility.
2. Use a 15 x 100 mm plate prepared according to SOP. Use a sterile 10  $\mu\text{l}$  inoculation loop to transfer bacteria from a plate or slant; use an aerosol resistant tip and pipetman to transfer cells from a liquid culture. A sterile cotton swap is ideal to spread the bacteria once they have been transferred to the plate.
3. Place inoculated plates in a Ziploc bag, seal, and place in warm room. Depending upon the strain, a lawn could take 2 to 4 weeks to form.
4. GAS + 0.05% tween is prepared according SOP M002.
5. The orbital platform should be rotating at approximately 200 RPM, enough to agitate the media so the cell clumps will disperse and the bacteria will be aerated.
6. Double check to make sure the Falcon cap is properly sealed and tightened before mixing.
7. Labels containing strain, lot number, date, medium, and technician name should be made before entering the BSL-3 facility, and placing them on the cryovials prior to bottling the cells. If the cryovials are frozen before label application, the labels will not adhere to the cryovials.
8. Use 100 x 15 mm plates prepared according to SOP M010.
9. Again, seal the plates in a Ziploc bag prior to incubation.