

SOP: PP002

Establishing frozen stocks of *Mycobacterium tuberculosis* (large scale)

Materials and Reagents:

1. *M. tuberculosis*, 1 ml frozen stock or growing culture (note 1)
2. Biosafety cabinet
3. 7H11 + OADC agar plate, large (15 x 150 mm) (note 2)
4. Inoculation loop, 10 μ 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 bag (note 3)
9. Cell scraper, sterile
10. GAS medium, 1000 ml (note 4)
11. Fernbach flask, 2800 ml, sterile (note 4)
12. Cotton and cheesecloth plug (note 4)
13. Foil
14. Bunsen burner
15. Orbital platform shaker
16. Pipet, 50 ml, sterile
17. Electric pipetter
18. Pipet boat, containing 10% Lysol I.C. solution
19. Falcon centrifuge tubes, 50 ml, sterile (4)
20. Parafilm
21. Freezer, -80°C
22. Autoclave

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. ____ Using a sterile cell scraper, aseptically transfer entire plate to one liter of GAS medium in a 2.8 liter fernbach flask (note 4).
5. ____ Gently flame the mouth of the fernbach flask, then aseptically replace the foil and plug.
6. ____ Incubate on an orbital platform shaker for 2 weeks at 37°C (note 5).
7. ____ Place the fernbach flask with the two week old bacterial pellet into the biosafety cabinet.
8. ____ Gently swirl the flask to dislodge cells from the sides of the flask and then set the flask down to allow the cells to settle.
9. ____ Once cells have settled, aseptically remove the foil and plug.
10. ____ Using a 50 ml pipet and electric pipetter, remove approximately $\frac{1}{4}$ of the bacterial pellet, along with 40 ml of medium (note 6).
11. ____ Aseptically transfer the cells and medium into a sterile 50 ml Falcon centrifuge tube (note 7).
12. ____ Gently flame the 50 ml Falcon tube and cap the tube.

13. ____ Repeat steps 10 to 12 with the remaining cell pellet.
14. ____ Discard the pipet into the pipet boat containing 10% Lysol I. C. solution.
15. ____ Replace the foil and plug on the fernbach flask.
16. ____ Label the four 50 ml Falcon tubes with the appropriate information (note 8).
17. ____ Seal the 50 ml Falcon caps to the tube with parafilm and snap freeze at -80°C .
18. ____ Autoclave the spent medium and flask, pipet boat and any remaining trash.

Notes:

1. Virulent strains of *M. tuberculosis* must be handled inside a BSL-3 facility.
2. Use a 15 x 150 mm plate prepared according to SOP M007. Use a sterile 10 μl 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 medium is prepared according SOP M001; medium should be placed inside the fernbach flask and the flask sealed with plug and foil prior to autoclaving.
5. The orbital platform should be rotating at approximately 100 RPM, enough to gently agitate the media to aerate the bacteria.
6. One fernbach flask will make 4 large stocks.
7. Make sure the total volume in the 50 ml Falcon tube is not greater than 40 ml, otherwise the tube may break during the freezing process.
8. Labels containing strain, lot number, date, medium, and technician name should be made before entering the BSL-3 facility, and placing them on the 50 ml Falcon tubes prior to bottling the cells. If the 50 ml Falcon tubes are frozen before label application, the labels will not adhere to the tubes.