SOP: PP033

Growth of *M.tb* for Mycobactin Production

Materials and Reagents

10% Lysol solution
100% ethanol
HR media (SOP M013)
M.tb 1 ml frozen stocks (Note 1)
HR agar plates (SOP M013)
Biosafety cabinet
Inoculation loops
P-1000 pipetor + sterile filter tips
Cryovials, 1.7 ml, with o-rings
Autopipetor
1 ml plastic serological pipets
Microcentrifuge
Pipet boat
Parafilm
Cell scrapers, 1.8 cm blade

Protocol

1._____Thaw as many 1 ml frozen stocks of *M. tuberculosis* as plates to be inoculated (Note 1).

2._____Transfer each stock to a separate cryovial with autopipetor and 1 ml pipets. Discard pipets into pipet boat filled with 10% Lysol solution (Note 2).

3._____Making certain caps are snug and parafilmed, spin tubes on low setting for 1 min.

4._____Inside the biosafety cabinet, use the P-1000 pipetor set to 800 µl to draw off the media, discard, and add 800 µl HR media (Note 3).

5._____Carefully resuspend the pellet by pipetting. Cap each tube before moving on to the next.

6._____Repeat steps (3) – (5) three times.

7._____Slowly pipet each tube of resuspended *M.tb* cells to a separate plate, ejecting tips into pipet boat. Spread the inoculum with sterile loops (Note 4).

8._____Transfer plates to large ziplock bag, getting rid of excess air, and place in warm room (Note 5).

9._____Once lawns are well established, transfer plates to biosafety cabinet and proceed to scrape plates. Carefully pull flat edge of instrument across the lawn in short strokes.

10._____Transfer cells as they accumulate on blade to bottle containing 50 ml 100% ethanol per plate to be harvested (Note 6).

11._____Swirl capped bottle of ethanol with harvested cells to ensure all cells are in ethanol solution.

12._____Place harvested plates back in bag used for growth, add several milliliters of 10% Lysol, then discard in biohazard bag in hood. Follow SOP for cleaning hood.

13._____Once the cells have sat in ethanol for at least 15 min after swirling, triple wrap bottle in medium biohazard bags, each containing a few milliliters of 10% Lysol (Note 7).

14._____Transport wrapped bottle back to BSL-2 facility and store at 4ºC until starting purification.

Notes

(1) Virulent *M. tuberculosis* must be handled inside a BSL-3 facility. A research associate stationed at the BSL-3 facility can thaw the cells a few hours before work is to begin.

(2) This will be to pellet cells for subsequent washing. Transfer all but a small volume of each culture to avoid aerosolization.

(3) HR media named after Hall and Ratledge, whose 1982 paper describes preparation of a growth media with no Fe, also used to make plates (see References). The original GAS/glycerol media will be drawn off the first time, and the pellet will be washed and resuspended in HR media.

(4) One technique is to turn plates 90º after spreading slowly from side to side. Ensure plates are completely covered with resuspension. Store each plate temporarily face-up and covered while working through them.

(5) Depending upon the strain, a lawn could take at least four weeks to grow. Consider flipping plates in three to four days to prevent drying. Colonies present at this time would likely be contaminating strains. The first *M.tb* colonies should appear in about one week, and will appear creamy white to beige, perhaps with a pink tint.

(6) Tip the bottle such that the mouth is over the plate to prevent dropping cells onto liner paper. Submerging spatula blade in the ethanol allows cells to come off readily.

(7) Apply Lysol to bottle liberally as it sits in first bag. Ensure each bag is securely taped.

References

RM Hall and C Ratledge (1982) A simple method for the production of mycobactin, the lipid-soluble siderophore, from mycobacteria. FEMS Microbiology Letters 15: 133-136.