SOP: PP049.1
4-15-20 A Simpson

Pellicle Growth of M. tuberculosis

Materials and Reagents:

1. *M. tuberculosis*, 1 ml frozen stock or growing culture (note 1)
2. Glass tube or glass bottle (s)
3. Inoculation loops or serological pipets
4. Glass Fernbach flask with plug and foil cover
5. Proskaur Beck medium (SOP M020, sterile filtered not autoclaved)
6. Glycerol, 80% stock solution or PB media with 20% glycerol
7. Bath Sonicator
8. Cryovials, cryovials box, and labels
9. Nutrient agars for sterility test
10. Spectrophotometer for OD600
11. CFU count supplies (SOP#?)
12. -80°C Freezer
13. Autoclave and BSL3 facility

Protocol:

1._____ Set up the biosafety cabinet inside the BLS3 (SP041). Thaw a 1mL frozen stock of *M. tuberculosis* or use a growing culture of *M. tuberculosis* (note 1).

2._____ Shake PB media to fully aerate prior to inoculation.

3._____ From 1mL of *M. tuberculosis* stock, transfer 1mL to a 10mL glass tube containing 5mL Proskaur and Beck (PB) medium. If using a loop of growth from a plate, can transfer directly to a larger volume (100mL PB medium in a glass bottle with a goodly amount of headspace). Gently float cells on the surface of the media.

4._____ Incubate the culture without agitation at 37°C. Pellicle will crash out of solution and cease to grow if disturbed. Check for growth regularly for the formation of a layer of organism growing on top of the medium, this is the pellicle. Formation of the pellicle layer will depend on the strain and container volume/type (note 2).

5._____ When the pellicle is confluent, transfer the pellicle using a sterile inoculation loop slightly bent on loop side or a detached serological pipet. Carefully transfer the pellicle to a larger glass container and gently lay on the surface of the media.

6._____ Incubate the culture at 37°C without agitation and observe for pellicle formation same as before.

7._____ Repeat the previous two steps to a preferred final volume. One Fernbach flask containing 1L of PB media works well.

8._____ Incubate the culture at 37°C without agitation and observe for pellicle formation same as before.

9._____ Harvest pellicle. For creating cryovials for storage, carefully transfer the pellicle to a 50 ml Falcon conical tube containing 40mL PB media with 20% glycerol.

10._____ Spin on a stir plate 1-2 days to fully disperse cells and break down clumps.

11._____ Bath sonicate the suspension 6 times for 30 seconds each with 30 seconds rest between.

12._____ If the previous two steps are insufficient, carefully push cells up and down through a 5mL syringe with an 18 gauge needle to break up any remaining clumps.

13._____ Aliquot into cryovials keeping all cells well suspended so maintain vial consistency.

14._____ Freeze stocks at -80°C for storage. Sterility test on nutrient agar. Check OD600 and do CFU plates.

Notes:

1. Virulent *M. tuberculosis* must be handled inside a BSL3 facility. One loop of growth from a plate works better than reviving a culture from a frozen stock.
2. The remaining medium below the pellicle should be clear with a little bit of growth on the bottom. Strains show different growth patterns, but the media should remain clear to slightly clear yellow as time progresses. If it is cloudy, this indicates most of the organism is dead or the culture is contaminated (if rapid). Time till confluent growth is highly variable (weeks to months).