SOP: PP055
Modified: 2-24-17 ASimpson

Large-scale growth of *Mycobacterium bovis*

Materials and Reagents:
1. *M. bovis*, 1 mL frozen stock
2. Biosafety cabinet (BSC)
3. Absorbent bench liner
4. Several Wypall wipes
5. Pipette boat half filled with 2.5% Vesphene solution
6. Extra gloves
7. Biosafety bag and holder for trash, autoclave tape
8. Squeeze bottle with 2.5% Vesphene solution and one with 70% ethanol
9. Lysol spray, Professional LYSOL BrandII Disinfectant Spray
10. Bunsen burner and striker (recommended on bottles/lids)
11. Large 7H11 + Sodium pyruvate agar plates, (15x150mm, SOP M018, give enough lead time for media prep)
12. Inoculation loops
13. P-200 pipettor
14. P-200 tips, sterile, aerosol-resistant tips
15. Ziploc bags
16. 2.8L glass fernbach flasks containing 1L sterile SPAS (SOP M014) medium, capped with cotton and cheesecloth plugs wrapped in aluminum foil, give enough lead time for media prep, need 4, then 16 every 3 weeks thereafter though final pass)
17. Cell scrapers
18. Parafilm
19. Orbital platform shaker
20. Several serological 50mL pipettes
21. Several serological 10mL pipettes
22. Serological pipettor
23. 4L Winchester bottles, sterile (autoclaved old, very clean, 4L brown bottles, 4/CFP harvest)
24. Several 0.2um VacuCap bottle filtration units
25. 230mL centrifuge bottle
26. Harvard trip balance
27. Centrifuge and Sorvall benchtop centrifuge rotor, containing 230mL centrifuge bottle hanging buckets and 2° plastic containment
28. Sterile Milli-Q water (autoclaved by media prep)
29. Warm rooms 102B/C BRB (37°C) storage of cultures during the incubation time and of things to be autoclaved
30. 4ºC cold room (BRB Molecular Biology room 101)
31. Rubbermaid transport cart
32. Vacuum pump and hosing setup
33. –80ºC freezer
34. Autoclave
35. Safety glasses and other PPE (personal protective equipment)

Protocol:
1. _____ Set up a BSC in the BRB (SOP SP041b) and thaw a 1mL frozen stock of *M. bovis*. One stock can make 3-5 plates.

2. _____ Pipette 200µL of the stock onto a large 7H11+Sodium pyruvate agar plate (SOP M018) and streak to grow as a lawn with a sterile bent plastic loop. Streak 3-5 large plates to later upscale to 4-5 fernbach flasks. Best to make extra.

3. _____ Place inoculated plates into Ziploc bags, seal, and place in the warm room. Depending upon the strain, a lawn could take 4 to 6 weeks to form. *M. bovis* is very slow growing.

4. _____ Take down the BSC (SOP SP041b).

5. _____ Incubate the plates at 37°C on a shelf in the warm room until a thick lawn has formed.

6. _____ Set up a BSC (SOP SP041b). Place the plates showing good growth and 4 fernbach flasks into the BSC.

7. _____ Remove a plate from the ziploc bag and scrape the cells into a pile. Using the cell scraper, aseptically transfer cells to a fernbach flask.

8. _____ Flame the mouth of the fernbach flask, then replace the cotton plug/foil.

9. _____ Repeat steps 7-8 with remaining plates and fernbach flasks.

10. _____ Take down the BSC (SOP SP041b).

11. _____ Incubate the fernbachs on an orbital platform shaker for 3 weeks at 37°C @ 55-65rpm.

Upscale:

12. _____ Three weeks later, set up the BSC (SOP SP041.1b), place 1 fernbach flask with good cell growth into the biosafety cabinet. *M. bovis* has clumpy flakes and flocculates well. The CFP is very thick though does not look cloudy.

13. _____ Gently swirl a flask and set it down to allow the cells to form into a pile in the center.

14. _____ Once the cells have settled, remove the cotton plug/foil.

15. _____ Using a 50mL pipette and serological pipettor, remove approximately $^1/_4$ of the bacterial pellet and transfer it to one of the new fernbach flasks. Re-swirl the flask if the cell pellet dissipates.

16. _____ Rest the pipette/pipettor in the fernbach flask containing the cells. Then, gently flame the top of the newly inoculated fernbach flask and replace the cotton plug/foil.

17. _____ Repeat steps 15-16 with remaining 3 flasks.

18. _____ Repeat steps 12-17 3x to inoculate the 12 remaining fernbach flasks. 4-5 fernbach flasks fit well in the hood at one time.

19. _____ Discard the pipette into the pipette boat containing a 2.5% Vesphene solution. Add 1/3 of the squeeze bottle containing 2.5% Vesphene solution to the used fernbach and replace the cotton plug/foil.

20. _____ Place the newly inoculated fernbach flasks on an orbital platform shaker at 37°C for three weeks.

21. _____ Take down the BSC (SOP SP041b). Leave up the BSC if harvesting cells and/or CFP.

Harvest protocol:

1. _____ Set up the BSC (SOP SP041b) if upscale was not done, otherwise continue from previous step.

2. _____ Transfer the 12-16 fernbach flasks from the shaker table to the cart.

3. _____ Place 4-5 fernbach flask cultures into the BSC and allow time for the cell pellets to settle.

4. _____ If harvesting CFP, place a sterile and clean 4L liter bottle, along with a VacuCap, into the BSC. Using autoclave tape, fasten the tubing from the vacuum pump to the BSC to prevent the tubing from causing accidental spillage. Turn on the vacuum pump and allow to warm-up for five minutes before using.

5. _____ Gently swirl several flasks to create a cell pellet to form in the center of each flask. Remove the cotton plug/foil from several fernbach flask cultures. Pipette the cell pellet from one flask into another flask. Use the flask containing only CFP as a catch for several containers worth of CFP. Use two flasks for this. Collect all the pellets into one flask with the pipettor. Gently rest the pipette/pipettor in the cell collection flask. Carefully pour the CFP from flasks into the CFP collection flask.

6. _____ Open the 4L bottle and VacuCap package. Snap off the cotton end of a 10mL pipette with the leverage of the handle of the Bunsen burner striker. Aseptically remove from plastic wrapper. Place the broken end into the flask containing CFP and insert the pointy end into the VacuCap hose. Flame the mouth of the bottle and place the VacuCap on it and attach the tubing from the vacuum pump to the VacuCap.

7. _____ Each bottle can hold 4L (so 4 bottles will be needed for harvest). Carefully watch the bottle begin to fill. If more than an inch of foam forms, the bottle was not rinsed correctly. Start over with a new bottle and discard old one and VacuCap. Also watch for crack formation due to the pressure.

8. _____ Remove the VacuCap from the 4L bottle when the bottle is full or if it is clogged (repeat step 6) and throw in trash bag in BSC. *M. bovis* CFP is very thick and uses more filters. Change filters when they get slow, they don't look clogged. Gently flame the mouth of the 4L bottle and cap.

9. _____ Add 2.5% Vesphene to empty fernbach flasks then cap with their foil and plugs. Using a 2.5% Vesphene solution or Lysol spray, wipe/spray down the empty fernbach flasks and remove from the biosafety cabinet and autoclave.

10. _____ Using the Lysol spray, spray down the 4L bottle and remove from the BSC.

11. _____ Repeat steps 6-15 until all pellets are in one collection flask and all CFP has been filtered, if CFP is harvested.

12. _____ Using the Rubbermaid transport cart, move the four 4L bottles containing sterile-filtered culture supernatant into the 4°C walk-in cold room. Take the CFP to main campus later. Add 5g sodium azide (NaN3) to the CFP if it will be in storage in Micro C222 4°C for more than a few days.

13. _____ Weigh an empty, sterile 230mL Polycarbonate conical then place it in a centrifuge bucket and place in the BSC (tends to weigh about 52.5g).

14. _____ Using a 50mL pipette and serological pipettor, transfer the bacterial cells into an empty flask.

15. _____Allow the cells to settle and pipette off the media into the collection flask.

16. _____Add 1Lsterile Milli-Q water to flask containing cells. Swirl flask to wash. Allow the cells to settle and pour off the supernatant.

17. _____Repeat step 16. Pipette cells into a 230mL conical with water and cap.

18. _____ Spray down and remove the Polycarbonate conical and bucket from the hood and place in the 2° centrifuge container.

19. _____ Turn on the benchtop centrifuge and fill in the user sheet.

20. _____ Use the Harvard trip balance and make a water balance for the pellet.

21. _____ Add or remove water as necessary to the water balance to balance the two Polycarbonate conicals.

22. _____ Once balanced, place the buckets onto the centrifuge rotor.

23. _____ Centrifuge at 3000rpm, 4^{o}C for 15 minutes.

24. _____ Remove the 230mL Polycarbonate conical containing the bacterial pellet from the centrifuge and place in the BSC.

25. _____ Carefully pipette the supernatant from the 230mL Polycarbonate conical into the collection flask. Be careful not to disturb the cell pellet.

26. _____ Squirt some Vesphene solution into the used fernbach flask and replace the cotton plug/foil.

27. _____ Spray down and remove the flask and the 230mL Polycarbonate conical from the BSC.

28. _____ Place the 230mL Polycarbonate conical on the Harvard trip balance and weigh (subtract from initial weight to get the final pellet wet weight). The empty 230mL Polycarbonate conicals tend to weigh 52.5g.

29. _____ Wrap the base of the cap with parafilm.

30. _____ Label the CFP and cell pellet with the appropriate information (strain, lot number, date, medium, and technician name) and parafilm the lids. Write down the weight of the cell pellet on the label and on the large scale growth worksheet. Package the 230mL Polycarbonate conical with pellet in three layers of biohazard bags with Lysol spray between layers 2 and 3. Seal each layer with tape. Label the outside of the package and freeze in the skinny -80 until the sample can be transported to Biochemistry for irradiation. The cell pellet is ready to be removed from the BSL-3 for γ-irradiation or frozen at –80^{o}C for DNA extraction. To remove the bacterial cell pellet from the BSL-3, please refer to the BRB Bulk Culture suite SOP manual. Alamar blue assay may be done with SPAS media. Need transport certification to transport infectious material. For DNA extraction see SOP PP009.2.

31. _____ Take down the BSC (SOP SP041b).

32. _____Repeat every three weeks through final pass (variable).

33. _____Note, CFP concentration will look odd. Salt will drop out of solution and form crystals due to the pyruvate in the SPAS media.