SOP: PP003.4
Modified: 7-24-23 AS

Large-scale growth of *Mycobacterium tuberculosis*

Materials and Reagents:

1. *M. tuberculosis*, 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
9. Lysol spray Professional LYSOL BrandII Disinfectant Spray
10. Bunsen burner and striker (recommended on bottles/lids)
11. 7H11 + OADC agar plate, large (15 x 150 mm, SOP M009, give enough lead time for media prep)
12. Inoculation loop, 10 µL
13. P-200 pipettor
14. P-200 tips, sterile, aerosol-resistant tips
15. Ziploc bags (3)
16. 2.8 L glass fernbach flasks containing 900 ml sterile GAS medium, capped with cotton and cheesecloth plugs wrapped in aluminum foil (SOP M001, give enough lead time for media prep, need 3, then 8 every 2 weeks thereafter though pass 9)
17. Cell scrapers
18. Parafilm
19. Orbital platform shaker
20. Several serological 50ml pipettes
21. Several serological 10ml pipettes
22. Serological pipettor
23. 1L rollerbottles containing 400 ml sterile GAS medium, (SOP M001, give enough lead time for media prep, need 40 every 2 weeks through pass 9)
24. Four 4 liter Winchester bottles, sterile (autoclaved old, very clean, 4L bottles)
25. Five 0.2 µm VacuCap bottle filtration units
26. 230 ml centrifuge bottle
27. Harvard trip balance
28. Centrifuge and Sorvall benchtop centrifuge rotor, sealed buckets with gaskets and 230 mL centrifuge bottle inserts
29. Sterile Milli-Q water (autoclaved by media prep)
30. Warm rooms 102B/C BRB (37ºC) storage of cultures during the incubation time and of things to be autoclaved
31. 4°C cold room (BRB Molecular Biology room 101)
32. Rollerbottle apparatus
33. Rubbermaid transport cart
34. Vacuum pump and hosing setup
35. –80ºC freezer
36. Autoclave
37. 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. tuberculosis*. One stock can make five plates.

2._____ Pipette 200 µL of the stock onto a large 7H11 + OADC agar plate (SOP M007) and streak to grow as a lawn with a sterile bent plastic loop. Streak at least three large plates for upscale to fernbach flasks.

3._____ Place inoculated plates into Ziploc bags, seal, and place in the warm room. Depending upon the strain, a lawn could take three to six weeks to form. Normally four weeks is sufficient.

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

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

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

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

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

9._____ Repeat the previous two steps with remaining plates and fernbach flasks.

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

11._____ Incubate the 3 fernbachs on an orbital platform shaker for 2 weeks (most strains) at 37ºC at ~65rpm.

Upscale:

12._____ Two weeks later, set up the BSC (SOP SP041b), place the three fernbach flasks with good cell growth into the biosafety cabinet. Note: some strains are more flocculating than others.

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, and flame the top.

15._____ Using a 50mL pipette and serological pipettor, remove approximately $^{1}/_{8}$ of the bacterial pellet and transfer it to one of the new fernbach flasks. Re-swirl the flask if the cell pile 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 the previous two steps on the seven remaining fernbach flasks. Four to five fernbach flasks fit well in the hood at one time.

18._____ 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.

19._____ Place the eight newly inoculated fernbach flasks on an orbital platform shaker at 37°C for two weeks

20._____ Place ten rollerbottles with 400mL of GAS medium inside the BSC. Rollerbottles are only used for strains where CFP is collected.

21._____ Place one fernbach flask with two week old bacterial growth inside the cabinet.

22._____ Swirl the flask gently to dislodge cells from the sides of the flask, and then set the flask down to allow the cells to settle.

23._____ Once the cells have settled, carefully remove the foil and cotton plug.

24._____ Using a 50mL pipette and serological pipettor, remove approximately $^{1}/_{20}$ of bacterial pellet and aseptically transfer it to one of the rollerbottles.

25._____ Gently flame the mouth of the rollerbottle and screw the cap on tightly.

26._____ Repeat the previous two steps on the nine remaining rollerbottles.

27._____ Remove the ten freshly inoculated rollerbottles and replace with 10 new rollerbottles containing GAS medium.

28._____ Repeat the previous steps for the ten new rollerbottles.

29._____ Discard the pipet into the pipet 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.

30._____ Remove the ten freshly inoculated rollerbottles from the biosafety cabinet. The rollerbottle caps should be double checked to ensure they are on correctly and tightly.

31._____ Repeat steps 20 through 30 on the remaining twenty rollerbottles.

32._____ Place the forty inoculated rollerbottles on rollerbottle apparatus at 37ºC for two weeks. Two rows can fit /level. Once placed on the apparatus, the rollerbottles should be rotated at a slow speed to ensure proper aeration of the cells while avoiding over-agitation.

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

Harvest protocol:

1._____ Set up the BSC (SOP **SP041b**).

2._____ Turn off the orbital platform shaker and rollerbottle apparatus and allow each to come to a complete stop.

3._____ Transfer the forty rollerbottle cultures to a cart.

4._____ Transfer the eight fernbach flasks to the cart. Save three flasks for inoculating more fernbach flasks and rollerbottles as described above.

5._____ Transport the laden cart from the warm room to the bulk culture room.

6._____ Place up to five fernbach flask cultures into the BSC and allow time for the cell pellets to settle.

7._____ 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.

8._____ 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. Collect all the pellets into one flask with the pipettor. Gently rest the pipette/pipettor in the cell

collection flask. If collecting CFP, collect from rollerbottles only unless otherwise requested. Use empty flasks as collection containers.

9._____ 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. Place the VacuCap on the mouth of the bottle and attach the tubing from the vacuum pump to the VacuCap.

10._____ Each bottle can hold 4L (so four to five 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.

11._____ Remove the VacuCap from the 4L bottle when the bottle is full or if the VacuCap is clogged and throw in trash bag in BSC. Gently flame the mouth of the 4L bottle and cap.

12._____ Cap all fernbach flasks with their foil and plugs. Using a 2.5% Vesphene solution or Lysol spray, wipe/spray down the four empty fernbach flasks and remove from the biosafety cabinet and autoclave.

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

14._____ Place a sterile 4L bottle, a VacuCap, and six-ten rollerbottle cultures inside the BSC. Continue filling the previous 4L bottle if it has space. Do not allow the VacuCap to dry out, if it does replace it with a new one. Lean the rollerbottles against the back wall and each other to allow the cells to settle in a pile.

15._____ Uncap a rollerbottle and keep it tilted slightly to allow cell pellet to stay settled. Occasionally, the sides of rollerbottle cultures will collapse as the internal temperature changes from 37ºC to 20°C. When this occurs, hold compressed area of rollerbottle with one hand while slowly opening the cap with the other. This will control the re-expansion of the rollerbottle, and prevent the aerosolizing of bacteria.

16._____ Pipette out the cell pellet and add it to the fernbach flask containing the other pellets. Carefully pour the media into the collection fernbach flask.

17._____ When empty, squirt in some Lysol and recap the rollerbottle. Repeat the previous two steps for the remaining rollerbottles.

18._____ When the bottle contains 4L of CFP, discard the VacuCap. Gently flame the mouth of the bottle and cap.

19._____ Spray down all empty rollerbottles and remove from the biosafety cabinet. Place empty rollerbottles inside two large autoclave bags for decontamination and disposal (~twenty/bag).

20._____ Spray down the 4L bottle and remove from biosafety cabinet.

21._____ Repeat the previous steps for the remaining thirty rollerbottle cultures. Using the Rubbermaid transport cart, move the 4L bottles containing sterile-filtered culture supernatant into the 4°C walk-in cold room. Add 5g sodium azide (NaN3) to the CFP if it will be in storage at 4ºC for more than a few days.

22._____ Weigh an empty, sterile 230mL Polycarbonate conical then place it in a centrifuge bucket in the BSC.

23._____ Using a 50mL pipette and serological pipettor, aseptically transfer the bacterial cells into the 230mL Polycarbonate conical. If there are more than 100g of cells, use two conicals.

24._____ When all of the cells have been transferred, dispose of the pipette into the pipette boat containing a 2.5% Vesphene solution.

25._____ Allow the cells in the conical to settle and pipette off the media.

26._____ Add sterile Milli-Q water up to the 200mL mark. Invert to mix the cells with the water. Allow the cells to settle and pipette off the supernatant.

27._____ Repeat the previous step several times then add sterile Milli-Q water to the 200mL mark.

28._____ Gently flame the mouth of the 230mL Polycarbonate conical and cap tightly.

29._____ Invert to mix the cells with the water.

30._____ Remove the Polycarbonate conical and bucket from the hood and place in the 2° centrifuge container.

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

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

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

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

35._____ Centrifuge at 3000rpm, 4^{o}C for 10-15 minutes.

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

37._____ Carefully pipette the supernatant from the 230mL Polycarbonate conical into the fernbach flask which had been used to hold the combined bacterial pellets. Be especially careful not to disturb the cell pellet.

38._____ Rewash and centrifuge the pellet.

39._____ Gently flame the mouth of the 230mL Polycarbonate conical and cap tightly.

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

41._____ Remove the flask and the 230mL Polycarbonate conical from the BSC.

42._____ Place the 230mL Polycarbonate conical on the Harvard trip balance and weigh (subtract from initial weight to get the final pellet wet weight).

43._____ 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. Thoroughly wrap the base of the cap with parafilm. Package the 230mL Polycarbonate conical with pellet in three layers of biohazard bags with Lysol spray between all layers. Seal each layer with tape. Label the outside of the package and freeze in the -80°C freezer 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°C for DNA extraction. To remove the bacterial cell pellet from the BSL-3, please refer toPP004.2..

Transport certification is required for transporting infectious material. For DNA extraction see SOP PP009.

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

45._____ Repeat upscale and harvest for several more passes (usually four harvests total).

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