

SOP: PP003.1

Modified: 8/31/2007

Large-scale growth of *Mycobacterium tuberculosis*

Materials and Reagents:

1. Biosafety cabinet (BSC)
2. Three 100 ml frozen stocks of *M. tuberculosis* (see SOP PP002)
3. Eight 2.8 L glass fernbach flasks containing 900 ml sterile GAS medium, capped with cotton and cheesecloth plugs wrapped in aluminum foil
4. Orbital platform shaker
5. Four 50 ml disposable pipets
6. Electric pipetter
7. Bunsen burner
8. Pipet boat
9. 10% Lysol I. C. solution
10. Autoclave
11. Forty 1 liter rollerbottles containing 400 ml sterile GAS medium
12. Two 25 ml disposable pipets
13. Rollerbottle apparatus
14. Rubbermaid transport cart
15. Vacuum pump
16. Five 4 liter Winchester bottles, sterile
17. Five 0.2 um Zap Cap S plus bottle filtration units
18. Five NaN₃ stocks
19. 225 ml Falcon centrifuge bottle
20. 225 ml centrifuge bottle rack
21. Harvard trip balance
22. Sorvall benchtop centrifuge
23. Sorvall benchtop centrifuge rotor, containing 225 ml centrifuge bottle hanging buckets
24. Sterile Milli-Q water
25. 4°C cold room (BHRB Molecular Biology room 101)
26. -80°C freezer (option, note 17)

Upscale protocol: (note 1)

1. ____ Inside a biosafety cabinet in a BSL-3 facility, thaw three “100 ml” stocks of *M. tuberculosis*.
2. ____ Carefully pour the contents of one stock into one 2.8 L fernbach flask containing GAS medium.
3. ____ Flame opening and aseptically replace foil and cotton plug in mouth of the fernbach flask.
4. ____ Repeat for two remaining “100 ml” stocks.
5. ____ Place the inoculated fernbach flasks on an orbital platform shaker at 37°C for two weeks (notes 2 and 3).
6. ____ Place four 2.8 L fernbach flasks with GAS medium inside the biosafety cabinet.
7. ____ Place one fernbach flask with a two week old bacterial growth inside the cabinet.
8. ____ 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.
9. ____ Once the cells have settled, aseptically remove the foil and cotton plug.

10. ____ Using a 50 ml pipet and electric pipetter, remove approximately $\frac{1}{8}$ of bacterial pellet and transfer it to one of the new fernbach flasks.
11. ____ Gently flame the fernbach opening and aseptically replace the cotton plug and foil In fernbach mouth.
12. ____ Repeat process on three remaining fernbach flasks.
13. ____ Discard the pipet into the pipet boat containing a 10% Lysol I. C. solution, and replace the cotton plug and foil on the old fernbach flask.
14. ____ Remove the four freshly inoculated fernbach flasks from the biosafety cabinet and replace with four 2.8 L fernbach flasks containing GAS medium.
15. ____ Repeat inoculation procedure for the four new fernbach flasks.
16. ____ Place the eight newly inoculated fernbach flasks on an orbital platform shaker at 37°C for two weeks (note 3).
17. ____ Autoclave the fernbach flask used for inoculation.
18. ____ Place 10 rollerbottles with 400 ml of GAS medium inside the biosafety cabinet.
19. ____ Place one fernbach flask with two week old bacterial growth inside the cabinet.
20. ____ 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.
21. ____ Once the cells have settled, aseptically remove the foil and cotton plug.
22. ____ Using a 25 ml pipet and electric pipetter, remove approximately $\frac{1}{20}$ of bacterial pellet and aseptically transfer it to one of the rollerbottles.
23. ____ Gently flame the mouth of the rollerbottle and replace the cap.
24. ____ Repeat steps 22 and 23 on the nine remaining rollerbottles.
25. ____ Discard the pipet into the pipet boat containing a 10% Lysol I. C. solution, and replace the cotton plug and foil on the used fernbach flask.
26. ____ Remove the 10 freshly inoculated rollerbottles and replace with 10 new rollerbottles containing GAS medium.
27. ____ Repeat steps 21 through 24 for the 10 new rollerbottles.
28. ____ Discard the pipet into the pipet boat containing a 10% Lysol I. C. solution, and replace the cotton plug and foil on the used fernbach flask.
29. ____ Remove the 10 freshly inoculated rollerbottles from the biosafety cabinet.
30. ____ Repeat steps 18 through 29.
31. ____ Place inoculated rollerbottles on rollerbottle apparatus at 37°C for two weeks (note 4).

32. ____ Autoclave the fernbach flasks used for inoculating the eight new fernbach flasks and forty new rollerbottles.

Harvest protocol: (note 1)

1. ____ Turn off the orbital platform shaker and rollerbottle apparatus and allow each to come to a complete stop.
2. ____ Transfer the 40 rollerbottle cultures onto a Rubbermaid transport cart.
3. ____ Check the fernbach flask cultures on the orbital platform shaker. Leave three fernbach flask cultures on the platform (those with the greatest cell density) and transfer the remaining five onto the Rubbermaid transport cart (note 5).
4. ____ Transport the cells from the warm room to the bulk culture room.
5. ____ Place the five fernbach flask cultures into the biosafety cabinet; allow time for the cell pellets to settle.
6. ____ Plug in the vacuum pump and turn on; allow to warm up for several minutes before attempting to pull a vacuum.
7. ____ Place a sterile four liter Winchester bottle, along with a Zap Cap S plus, into the biosafety cabinet (note 6).
8. ____ Open the Winchester bottle and Zap Cap package, and place the Zap Cap on the mouth of the bottle. Attach the tubing from the vacuum pump to the Zap Cap.
9. ____ Aseptically remove the cotton plug and foil from one fernbach flask culture, and tilt the flask slightly to allow cells to pellet away from the culture supernatant (note 7).
10. ____ Carefully pour the culture supernatant into the Zap Cap, trying to keep as many cells as possible inside the fernbach flask (note 8).
11. ____ Pour as much culture supernatant as possible from the fernbach flask, and set it down in the hood, loosely recap the flask. Use this flask to collect cells during the harvest.
12. ____ Repeat steps 9 and 10 with the remaining four fernbach flask cultures. Leave approximately 100 ml of medium in the flask to aid in the transfer of cells to the fernbach flask containing the combined bacterial pellets (notes 9 and 10).
13. ____ Remove the Zap Cap from the Winchester bottle and throw in trash bag in BSC. Add entire contents of a NaN_3 stock to the Winchester bottle, and throw stock tube into trash bag in BSC. Gently flame the mouth of the Winchester bottle and cap.
14. ____ Cap all fernbachs with their foil and plugs. Using a 10 % Lysol I. C. solution, wipe down the four empty fernbachs and remove from the biosafety cabinet and autoclave.
15. ____ Using the 10% Lysol I. C. solution, wipe down the Winchester bottle and remove from the biosafety cabinet.
16. ____ Place a sterile Winchester bottle, a Zap Cap and 10 rollerbottle cultures inside the biosafety cabinet.
17. ____ Attach the Zap Cap to the Winchester bottle and the vacuum pump tubing as previously described.

18. ____ Aseptically uncap a rollerbottle and tilt slightly to allow cell pellet to settle from medium (note 11).
19. ____ Pour the culture supernatant into the Zap Cap, leaving behind approximately 30 ml and as many cells as possible. Use the remaining medium to re-suspend the cells and transfer to the fernbach flask containing the combined bacterial pellets.
20. ____ When empty, cap the rollerbottle and repeat steps 18 and 19 for the remaining nine rollerbottle cultures.
21. ____ Remove the Zap Cap from the Winchester bottle and throw in trash bag in the biosafety cabinet. Add the entire contents of a NaN_3 stock to the Winchester bottle, and throw stock tube into the same trash bag. Gently flame the mouth of the Winchester bottle and cap.
22. ____ Using a 10 % Lysol I. C. solution, wipe down all empty rollerbottles and remove from the biosafety cabinet and autoclave (note 12).
23. ____ Using the 10% Lysol I. C. solution, wipe down the Winchester bottle and remove from biosafety cabinet.
24. ____ Repeat steps 16 through 23 for the remaining thirty rollerbottle cultures (note 13).
25. ____ Aseptically replace the cotton plug and foil on the fernbach flask containing the combined bacterial cells from the 21 L previously harvested.
26. ____ Place the flask down in the biosafety cabinet and allow the cells to settle out. Aseptically remove the cotton plug and foil.
27. ____ Place a sterile Falcon 225ml centrifuge bottle in the biosafety cabinet.
28. ____ Using a 50 ml pipet and electric pipettor, aseptically transfer the bacterial cells into the 225 ml Falcon bottle (note 14).
29. ____ When all of the cells have been transferred, dispose of the pipet into the pipet boat containing a 10% Lysol I. C. solution.
30. ____ Gently flame the mouth of the Falcon 225 ml centrifuge bottle and cap.
31. ____ Wipe down the centrifuge bottle with a 10% Lysol I. C. solution and remove from the biosafety cabinet.
32. ____ Turn on the benchtop Sorvall centrifuge and open the lid.
33. ____ Remove the 225 ml buckets from the rotor and place on the Harvard trip balance.
34. ____ Place the 225 ml centrifuge bottle containing the bacterial pellet in one of the rotor buckets.
35. ____ Place the 225 ml centrifuge bottle designated as the balance in the other rotor bucket (note 15).
36. ____ Add or remove water as necessary to balance the two centrifuge bottles.
37. ____ Once balanced, attach the splash guards to the centrifuge buckets and place the buckets onto the centrifuge rotor.

38. ____ Centrifuge at 3000 RPM, 4°C for 10 minutes.
39. ____ Remove the 225 ml centrifuge bottle containing the bacterial pellet from the centrifuge and place in the biosafety cabinet.
40. ____ Carefully decant the supernatant into the fernbach flask which had been used to hold the combined bacterial pellets. Be especially careful not to disturb the cell pellet.
41. ____ Add sterile Milli-Q water to the 225 ml centrifuge bottle, bringing the final volume to 200 ml.
42. ____ Gently flame the mouth of the 225 ml centrifuge bottle and cap.
43. ____ Wipe down the 225 ml centrifuge bottle with a 10% Lysol I. C. solution, and repeat steps 33 through 40.
44. ____ Gently flame the mouth of the 225 ml centrifuge bottle and cap.
45. ____ Thoroughly wrap the base of the cap with parafilm.
46. ____ Replace the cotton plug and foil in the mouth of the fernbach flask.
47. ____ Wipe down the 225 ml centrifuge bottle and fernbach flask with a 10% Lysol I. C. solution. Remove both from the biosafety cabinet.
48. ____ Place the 225 ml centrifuge bottle on the Harvard trip balance and weigh (note 16).
49. ____ Write down the weight on the bottle label and large-scale growth worksheet. The cell pellet is ready to be removed from the BSL-3 for γ -irradiation or frozen at -80°C for DNA extraction (note 17).
50. ____ Remove the pipet boat and trash from the biosafety cabinet.
51. ____ Autoclave all materials used for harvest.

Notes:

1. For convenience, large-scale growth is divided into upscale and harvest sections. Once large-scale growth is started and becomes a continuous process every other week, it is easier to harvest before upscale.
2. Steps 1 to 5 are used to initially start large scale growth of *M. tuberculosis*, or to keep large scale growth continuous after inoculation of passage 10. These five steps may be omitted when inoculum is from an actively growing culture.
3. Orbital platform shakers should be rotating only enough to produce a gentle swirling motion on the media and cells inside the fernbach. Typically, this is approximately 100 RPM.
4. The rollerbottle caps should be double checked to ensure they are on correctly and tightly; preferably the first check should be done inside the BSC. 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. Typically, this speed is 6.5 on the apparatus dial.
5. The three cultures left on the platform shaker will be used to inoculate more fernbach flasks and rollerbottles To ensure the best possible growth of new culture, it is advisable to inoculate with a culture that contains the greatest density of cells possible.
6. 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.
7. Use caution when tilting the fernbach to prevent aerosolizing the bacteria.
8. When pouring the culture supernatant into the Zap Cap, hold the Zap Cap to prevent it from tipping over and spilling. Also, pay close attention to small droplets which may splash when the culture supernatant is

poured into the Zap Cap; using a 10 % Lysol I. C. solution to mop up any droplets that land outside the Zap Cap.

9. If the Zap Cap dries between runs, carefully add sterile dH₂O before adding new filtrate. Caution should be used when combining pellets to avoid any possible aerosolization of bacteria.

10. If the Zap Cap becomes plugged with cells and filtering is too slowly, it may be changed for a new one between fernbach flasks.

11. Occasionally, the sides of rollerbottle cultures will suck in as the internal temperature changes from 37°C 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.

12. Place empty rollerbottles inside a 24" x 36" autoclave bag. Each bag can hold 13 rollerbottles. Put the last rollerbottle inside a 12" x 24" autoclave bag.

13. Using the Rubbermaid transport cart, move the five Winchester bottles containing sterile-filtered culture supernatant into the 4°C walk-in cold room in room 101, where it will be concentrated by Amicon filtration; please see Amicon filtration (see SOP PP005).

14. When the volume approaches 200 ml, allow the cells to settle and carefully pour the supernatant into the fernbach flask used to collect the combined bacterial pellet. Gently swirl the flask as previously described to re-pellet the bacteria.

15. The 225 ml centrifuge bottle contains numerous glass beads and is labeled "BALANCE." It always contains some volume of tap water.

16. Through years of meticulous research, it has been determined that the average weight of a Falcon 225 ml centrifuge bottle is 33.1 g.

17. To remove the bacterial cell pellet from the BSL-3, please refer to the BHRB Bulk Culture suite SOP manual. For using the bacterial cell pellet for DNA extraction see SOP PP009.