

Large Scale Growth Production Manual

Version 1
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SOP: PP001**Establishment of frozen stocks of *Mycobacterium tuberculosis*, small scale****Materials and Reagents:**

1. *M. tuberculosis*, 1 ml frozen stock or growing culture (note 1)
2. Biosafety cabinet
3. 7H11 + OADC agar plate, small (10 x 150 mm) (note 2)
4. Inoculation loop, 10 μ l (note 2)
5. P-1000 pipettor (note 2)
6. P-1000 tips, sterile, aerosol-resistant tips (note 2)
7. Cotton swab, sterile (note 2)
8. Ziploc bags
9. GAS medium + 0.05% tween, 100 ml (note 4)
10. Disposable Corning Erlenmeyer flask, 250ml, sterile
11. Cell scraper, sterile
12. Bunsen burner
13. Orbital platform shaker
14. Pipet, 5 ml, sterile
15. Electric pipettor
16. Sterile glass tube with cap (16 x 100 mm)
17. Spectrophotometer, visible light
18. Pipet boat, containing 10% Lysol I.C. solution
19. Sterile Falcon centrifuge tube (50 ml)
20. 80 % glycerol stock solution, sterile
21. Pipet, 10ml, sterile
22. Cryovials, 1.7 ml, sterile (100) (note 7)
23. Cryostorage box, 100-place
24. Freezer, -80°C
25. Autoclave
26. Nutrient agar plates, (10 x 150mm) (note 8)

Protocol:

1. ____ Thaw a 1 ml frozen stock of *M. tuberculosis* or obtain a growing culture of *M. tuberculosis* (note 1).
2. ____ Streak a small 7H11 + OADC agar plate with bacteria and spread to grow as a lawn (note 2).
3. ____ Incubate at 37°C until a lawn has formed (note 3).
4. ____ Inside the biosafety cabinet, use a sterile cell scraper and aseptically transfer several half of lawn to 100 ml of GAS + 0.05% tween in a 250 ml Corning Erlenmeyer flask (note 4).
5. ____ Gently flame the mouth of the Erlenmeyer flask and aseptically replace the lid.
6. ____ Incubate on an orbital platform shaker for 2 weeks at 37°C (note 5).
7. ____ Transfer the culture from the warm room into the biosafety cabinet.
8. ____ Using a sterile 5 ml pipet and an electric pipettor, transfer 5 ml of suspended cells to a sterile 16 x 100 mm glass tube and measure the O.D. A600; record in stock notebook. Discard the pipet into a pipet boat filled with 10% Lysol I. C. solution.
9. ____ Aseptically add 12.5 ml of sterile 80% glycerol stock solution into a sterile 50 ml Falcon centrifuge tube.

10. ____ Aseptically add 37.5 ml of suspended cells into the Falcon tube containing the glycerol stock solution and cap the tube.
11. ____ Mix thoroughly by inverting several times (note 6).
12. ____ Using a sterile 10 ml pipet, aseptically add 1 ml of cell suspension to each sterile 1.7 ml cryovial. Discard the pipet as before when finished (note 7).
13. ____ Cap the cryovials, place in a cryostorage box, and snap-freeze at -80°C .
14. ____ Autoclave spent media, 16 x 100 mm tube with cells, trash, and pipet boat.
15. ____ After 1 or 2 weeks, thaw several stocks.
16. ____ Streak stocks individually on a small nutrient agar plates and grow as a lawn (note 8).
17. ____ Incubate at 37°C and check for rapid growth of a possible contaminant. If no contaminant is present after 2 weeks, the stocks are ready to be included in the inventory (note 9).

Notes:

1. Virulent *M. tuberculosis* must be handled inside a BSL-3 facility.
2. Use a 15 x 100 mm plate prepared according to SOP. Use a sterile 10 μl inoculation loop to transfer bacteria from a plate or slant; use an aerosol resistant tip and pipetman to transfer cells from a liquid culture. A sterile cotton swap is ideal to spread the bacteria once they have been transferred to the plate.
3. Place inoculated plates in a Ziploc bag, seal, and place in warm room. Depending upon the strain, a lawn could take 2 to 4 weeks to form.
4. GAS + 0.05% tween is prepared according SOP M002.
5. The orbital platform should be rotating at approximately 200 RPM, enough to agitate the media so the cell clumps will disperse and the bacteria will be aerated.
6. Double check to make sure the Falcon cap is properly sealed and tightened before mixing.
7. Labels containing strain, lot number, date, medium, and technician name should be made before entering the BSL-3 facility, and placing them on the cryovials prior to bottling the cells. If the cryovials are frozen before label application, the labels will not adhere to the cryovials.
8. Use 100 x 15 mm plates prepared according to SOP M010.
9. Again, seal the plates in a Ziploc bag prior to incubation.

SOP: PP002

Establishing frozen stocks of *Mycobacterium tuberculosis* (large scale)

Materials and Reagents:

1. *M. tuberculosis*, 1 ml frozen stock or growing culture (note 1)
2. Biosafety cabinet
3. 7H11 + OADC agar plate, large (15 x 150 mm) (note 2)
4. Inoculation loop, 10 μ l (note 2)
5. P-1000 pipettor (note 2)
6. P-1000 tips, sterile, aerosol-resistant tips (note 2)
7. Cotton swab, sterile (note 2)
8. Ziploc bag (note 3)
9. Cell scraper, sterile
10. GAS medium, 1000 ml (note 4)
11. Fernbach flask, 2800 ml, sterile (note 4)
12. Cotton and cheesecloth plug (note 4)
13. Foil
14. Bunsen burner
15. Orbital platform shaker
16. Pipet, 50 ml, sterile
17. Electric pipetter
18. Pipet boat, containing 10% Lysol I.C. solution
19. Falcon centrifuge tubes, 50 ml, sterile (4)
20. Parafilm
21. Freezer, -80°C
22. Autoclave

Protocol:

1. ____ Thaw a 1 ml frozen stock of *M. tuberculosis* or obtain a growing culture of *M. tuberculosis* (note 1).
2. ____ Streak a small 7H11 + OADC agar plate with bacteria and spread to grow as a lawn (note 2).
3. ____ Incubate at 37°C until a lawn has formed (note 3).
4. ____ Using a sterile cell scraper, aseptically transfer entire plate to one liter of GAS medium in a 2.8 liter fernbach flask (note 4).
5. ____ Gently flame the mouth of the fernbach flask, then aseptically replace the foil and plug.
6. ____ Incubate on an orbital platform shaker for 2 weeks at 37°C (note 5).
7. ____ Place the fernbach flask with the two week old bacterial pellet into the biosafety cabinet.
8. ____ Gently swirl the flask to dislodge cells from the sides of the flask and the set the flask down to allow the cells to settle.
9. ____ Once cells have settled, aseptically remove the foil and plug.
10. ____ Using a 50 ml pipet and electric pipetter, remove approximately $\frac{1}{4}$ of the bacterial pellet, along with 40 ml of medium (note 6).
11. ____ Aseptically transfer the cells and medium into a sterile 50 ml Falcon centrifuge tube (note7).
12. ____ Gently flame the 50 ml Falcon tube and cap the tube.

13. ____ Repeat steps 10 to 12 with the remaining cell pellet.
14. ____ Discard the pipet into the pipet boat containing 10% Lysol I. C. solution.
15. ____ Replace the foil and plug on the fernbach flask.
16. ____ Label the four 50 ml Falcon tubes with the appropriate information (note 8).
17. ____ Seal the 50 ml Falcon caps to the tube with parafilm and snap freeze at -80°C .
18. ____ Autoclave the spent medium and flask, pipet boat and any remaining trash.

Notes:

1. Virulent strains of *M. tuberculosis* must be handled inside a BSL-3 facility.
2. Use a 15 x 150 mm plate prepared according to SOP M007. Use a sterile 10 μl inoculation loop to transfer bacteria from a plate or slant; use an aerosol resistant tip and pipetman to transfer cells from a liquid culture. A sterile cotton swap is ideal to spread the bacteria once they have been transferred to the plate.
3. Place inoculated plates in a Ziploc bag, seal, and place in warm room. Depending upon the strain, a lawn could take 2 to 4 weeks to form.
4. GAS medium is prepared according SOP M001; medium should be placed inside the fernbach flask and the flask sealed with plug and foil prior to autoclaving.
5. The orbital platform should be rotating at approximately 100 RPM, enough to gently agitate the media to aerate the bacteria.
6. One fernbach flask will make 4 large stocks.
7. Make sure the total volume in the 50 ml Falcon tube is not greater than 40 ml, otherwise the tube may break during the freezing process.
8. Labels containing strain, lot number, date, medium, and technician name should be made before entering the BSL-3 facility, and placing them on the 50 ml Falcon tubes prior to bottling the cells. If the 50 ml Falcon tubes are frozen before label application, the labels will not adhere to the tubes.

SOP: PP003

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, leaving the flask uncapped. 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 50 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. Make sure the Zap Cap does not go dry between fernbach flasks, as the filter will no longer be viable, and a new Zap Cap will need to be used. 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.

SOP: M001

Preparation of GAS medium protocol

Materials and Reagents:

1. Milli-Q water
2. Beaker, 1 liter
3. Magnetic stir bar
4. Magnetic stir plate
5. BactoCasitone (BD Science BD225930)
6. Ferric ammonium citrate (Sigma F-5879)
7. Potassium phosphate, dibasic anhydrous (VWR MK709208) (note 1)
8. Citric acid, anhydrous (VWR JT0122-1)
9. L-Alanine (Sigma A-7627)
10. Magnesium chloride, heptahydrate (VWR MK595804)
11. Potassium sulfate (VWR MK714004)
12. Ammonium chloride (VWR MK338412)
13. Sodium hydroxide, 10 M
14. Glycerol (VWR IC800689)
15. Graduated cylinder, 1 liter
16. Autoclave

Protocol:

1. ____ Pour 800 ml of Milli-Q water into a 1 liter beaker.
2. ____ Add magnetic stir bar to beaker and place on magnetic stir plate.
3. ____ Add 0.3 g of BactoCasitone.
4. ____ Add 0.05 g of ferric ammonium citrate.
5. ____ Add 4.0 g of potassium phosphate, dibasic anhydrous (note 1).
6. ____ Add 2.0 g of citric acid.
7. ____ Add 1.0 g of L-alanine.
8. ____ Add 1.2 g of magnesium chloride.
9. ____ Add 0.6 g of potassium sulfate.
10. ____ Add 2.0 g of ammonium chloride.
11. ____ Make sure all components are completely in solution.
12. ____ Add 1.8 ml of 10 M sodium hydroxide.
13. ____ Make sure the sodium hydroxide is completely in solution.
14. ____ Add 10.0 ml of glycerol.
15. ____ Make sure the glycerol is fully dispersed.
16. ____ Measure the pH, and adjust to 6.6

17. _____ Pour medium into 1 liter graduated cylinder.
18. _____ Bring volume to 1 liter with Milli-Q water.
19. _____ Transfer/aliquot to desired container(s).
20. _____ Autoclave on liquid cycle (slow exhaust) at 121°C for 15 minutes.

Notes:

1. **MUST** use Mallinckrodt K₂HPO₄; other brands will salt out during sterilization.

Reference:

Takayama, K., H. K. Schnoes, E. L. Armstrong, and R. W. Boyle. 1975. Site of inhibitory action of isoniazid in the synthesis of mycolic acids in *Mycobacterium tuberculosis*. *J. Lipid Res.* 16: 308-317.

SOP: M002

Preparation of GAS medium + 0.05% Tween 80 protocol

Materials and Reagents:

1. Milli-Q water
2. Beaker, 1 liter
3. Magnetic stir bar
4. Magnetic stir plate
5. BactoCasitone (BD Science BD225930)
6. Ferric ammonium citrate (Sigma F-5879)
7. Potassium phosphate, dibasic anhydrous (VWR MK709208) (note 1)
8. Citric acid, anhydrous (VWR JT0122-1)
9. L-Alanine (Sigma A-7627)
10. Magnesium chloride, heptahydrate (VWR MK595804)
11. Potassium sulfate (VWR MK714004)
12. Ammonium chloride (VWR MK338412)
13. Tween 80 (Fisher T164-500), 20% solution, sterile
14. Sodium hydroxide, 10 M
15. Glycerol (VWR IC800689)
16. Graduated cylinder, 1 liter
17. Autoclave

Protocol:

1. ____ Pour 800 ml of Milli-Q water into a 1 liter beaker.
2. ____ Add magnetic stir bar to beaker and place on stir plate.
3. ____ Add 0.3 g of BactoCasitone.
4. ____ Add 0.05 g of ferric ammonium citrate.
5. ____ Add 4.0 g of potassium phosphate, dibasic anhydrous (note 1).
6. ____ Add 2.0 g of citric acid.
7. ____ Add 1.0 g of L-alanine.
8. ____ Add 1.2 g of magnesium chloride.
9. ____ Add 0.6 g of potassium sulfate.
10. ____ Add 2.0 g of ammonium chloride.
11. ____ Make sure all components are completely in solution.
12. ____ Add 1.8 ml of 10 M sodium hydroxide.
13. ____ Make sure the sodium hydroxide is completely in solution.
14. ____ Add 10.0 ml of glycerol.
15. ____ Make sure the glycerol is fully dispersed.
16. ____ Add 2.5 ml of 20% Tween solution to make a final Tween concentration of 0.05%.

17. _____ Measure the pH, and adjust to 6.6.
18. _____ Pour medium into 1 liter graduated cylinder.
19. _____ Bring volume to 1 liter with Milli-Q water.
20. _____ Transfer/aliquot to desired container(s).
21. _____ Autoclave on liquid cycle (slow exhaust) at 121°C for 15 minutes (note 2).

Notes:

1. **MUST** use Mallinckrodt K_2HPO_4 ; other brands will salt out during sterilization.
2. The medium will appear cloudy immediately after sterilization, but will clear upon cooling.

Reference:

Takayama, K., H. K. Schnoes, E. L. Armstrong, and R. W. Boyle. 1975. Site of inhibitory action of isoniazid in the synthesis of mycolic acids in *Mycobacterium tuberculosis*. *J. Lipid Res.* 16: 308-317.

SOP: M007

Preparation of Middlebrook 7H9-Dextrose broth protocol

Materials and Reagents:

1. Milli-Q water
2. Beaker, 1 liter
3. Magnetic stir bar
4. Magnetic stir plate
5. Middlebrook 7H9 broth (VWR 90003-876)
6. Dextrose (VWR 90000-908)
7. Glycerol (VWR IC800689)
8. Graduated cylinder, 1 liter
9. Autoclave

Protocol:

1. ____ Pour 700 ml of Milli-Q water into a 1 liter beaker.
2. ____ Add magnetic stir bar to beaker and place on magnetic stir plate.
3. ____ Add 4.7 g of Middlebrook 7H9 dehydrated broth.
4. ____ Add 2.0 g of Dextrose to make a final concentration of 2% (note 1).
4. ____ Make sure all components are completely in solution.
5. ____ Add 2 ml of glycerol.
6. ____ Make sure the glycerol is fully dispersed.
7. ____ Pour medium into 1 liter graduated cylinder.
8. ____ Bring volume to 900 ml with Milli-Q water.
9. ____ Transfer/aliquot to desired container(s).
10. ____ Autoclave on liquid cycle (slow exhaust) at 121°C for 15 minutes.

Notes:

1. This is the amount of dextrose present when OADC is added to the medium at a final concentration of 10%. Therefore, OADC does not need to be added.

Reference:

Diffco manual, 10th edition. 1984 Difco Lab, Inc. Detroit, MI 48232.

SOP: M010

Preparation of Middlebrook 7H11-Dextrose agar protocol

Materials and Reagents:

1. Milli-Q water
2. Beaker, 1 liter
3. Magnetic stir bar
4. Magnetic stir plate
5. Middlebrook 7H11 agar (VWR 90004-942)
6. Dextrose (VWR 90000-908)
7. Glycerol (VWR IC800689)
8. Graduated cylinder, 1 liter
9. Autoclave
10. Water bath, 55°C
11. Serological pipet, 50 ml, sterile
12. Electric pipettor
13. Sterile plates, 15 x 150 mm or 15 x 100 mm.
14. Serological pipet, 10 ml, sterile
15. Sharpie marker
16. Ziploc bag, one gallon

Protocol:

1. ____ Pour 700 ml of Milli-Q water into a 1 liter beaker.
2. ____ Add magnetic stir bar to beaker and place on magnetic stir plate.
3. ____ Add 21.0 g of Middlebrook 7H11 dehydrated agar.
4. ____ Add 2.0 g of dextrose to make a final concentration of 2% (note 1)
4. ____ Make sure all components are completely in solution.
5. ____ Add 5 ml of glycerol.
6. ____ Make sure the glycerol is fully dispersed.
7. ____ Pour medium into 1 liter graduated cylinder.
8. ____ Bring volume to 900 ml with Milli-Q water.
9. ____ Transfer/aliquot to desired container(s) (note 2).
10. ____ Autoclave on liquid cycle (slow exhaust) at 121°C for 15 minutes.
11. ____ Place sterile medium in 55°C water bath for 30 minutes (note 3).
12. ____ Pour agar into plates (note 4).
13. ____ Remove any bubbles on plates by pipetting with a 10 ml pipet and electric pipettor.
14. ____ Allow plates to cool and solidify.
15. ____ Label plates and store at 4°C in a Ziploc bag.

Notes:

1. This is the amount of dextrose present when OADC is added to the medium at a final concentration of 10%. Therefore, OADC does not need to be added.
2. It is best to make up the desired amount of agar in each container/volume desired instead of making aliquots from a 900 ml stock; otherwise, the solution needs to be brought to a boil to completely re-suspend the agar prior to making aliquots. If the agar is not boiled, then it will be unevenly dispersed between containers and the plates will not solidify correctly.
3. Allows the agar solution to cool to a temperature that allows for handling without solidifying the agar.
4. One batch of 7H11 agar with dextrose will make approximate six 15 x 150 mm plates or thirteen 15 x 100 mm plates. Plates should be poured thickly to ensure they do not completely dry out when used for culturing of *M. tuberculosis*.

Reference:

Difco manual, 10th edition. 1984 Difco Lab, Inc. Detroit, MI 48232.