PP050.1
Modified: 1/20/2015 by AGA

Purification of M.leprae from armadillo tissue

Materials and Regents

1. Infected liver or spleen tissue (note 1)
2. 1 x 1L sterile beaker (note 2)
3. 1 x 400 ml sterile beaker (note 2)
4. 4 x 250 ml sterile beakers (note 2)
5. 14 x 50 ml Teflon Oakridge (Nalgene Cat # 314-0050) tubes (note 2)
6. Homogenization chamber
7. Homogenizer
8. 10 mM Na_2EDTA (note 3)
9. Sterile stir bars
10. Sterile scissors
11. BSL-2 hood
12. Brain Heart Infusion (BHI) broth
13. Milli-Q water
14. Tryptic Soy Agar (TSA)
15. 100 mm Petri plates
16. Inoculation loop
17. Flame or heat source
18. Centrifuge
19. Balance
20. Parafilm
21. Ice
22. Ice bucket
23. Stir plate
24. 10 mM Na_2EDTA/ 0.1 M NaOH buffer (note 3)
25. 0.1 M Tris/HCL buffer (note 3)
26. 37°C incubator
27. 0.2 µm Acrodisc syringe filters
28. 1 cc syringes
29. 500 ml separatory funnel
30. Stop cock
31. Stand
32. 25 ml pipet
33. 50 ml pipet
34. 0.2% Tween 80 solution (note 3)
35. 4.0 ml cryovials
36. Microslides
37. Microscope
38. Glass tubes
39. Spectrometer
40. Tube rack
41. Buffer water (note 3)
42. 1 M Calcium chloride (note 3)
43. 6% PEG/8% Dextran/9% Phosphate saline buffer (note 3)

Protocol

Day 1

1._____ Remove tissue from -80°C freezer at the end of the day, place in a sterile 250 ml beaker. Recover beaker with foil and place in 4°C overnight to thaw.

Day 2 (work in a BSL2 hood to minimize sample contamination and spray/wipe outsides of tubes with Lysol every time they leave the hood)

2._____ Aseptically chop tissue with scissors into approximately 1 cm pieces and pour into sterile homogenization chamber.

3._____ Rinse container with cold 10 mM Na_2EDTA and add to homogenization chamber. Add 10 mM Na_2EDTA to the chamber to a final volume of 30 ml per 10 g of tissue.

4._____ Homogenize tissue on ice using setting 5.5 for 3 min (note 4).

5._____ Pour homogenate into sterile oakridge tubes, balance and centrifuge at 500 x g at 4°C for 5 min.

6._____ While centrifuging, perform a sterility test with the small amount of homogenate left in the chamber. To do this, flame an inoculation loop, wait ~10 seconds to cool, take a loop full of homogenate and streak a LJ slant. Repeat the process and inoculate a TSA plate and BHI media (parafilm the TSA plate to ensure that agar doesn't dry out). Incubate all three at 37°C (note 5).

7._____ Pour supernatant into sterile 250 ml beaker with a stir bar and stir on medium speed to maintain homogenization.

8._____ Resuspend pellet by vortexing in a minimal amount of Na_2EDTA buffer (approx. 20 ml) and pour into the previously used homogenization chamber. Homogenize as in step 4.

9._____ Combine homogenate with the supernatant. Rinse chamber with ~5 ml of 10 mM Na_2EDTA buffer and add to homogenate/supernatant combination. Continue to stir for a ~2 min to decrease the amount of foam layer on the top. Pour homogenate into the same 50 ml Oakridge centrifuge tube as before, being sure to avoid pouring the stir bar into tubes (note 6). More tubes may be used if needed. Centrifuge at 4°C for 10 min at 10,000 x g.

10._____ Pour supernatant into a 280 ml conical bottle, label with date, volume and tissue type, store at -20°C for PGL-I extraction (see SOP:PP052).

11._____ Resuspend pellet by vortexing in 5 ml/tube of 10 mM Na_2EDTA/ 0.1 M NaOH buffer and pour into a sterile 250 ml beaker. Rinse tube(s) 3x by vortexing and combine with resuspended pellet. Adjust volume to ~200 ml and stir with stir bar on ice for 2 hrs. (Suspension may become viscous at this stage)

12._____ Pour suspension into the same 50 ml Oakridge centrifuge tube and centrifuge at 10,000 x g for 10min at 4°C. Discard supernatant as a biohazard.

13._____ Resuspend pellet by vortexing in 0.1 M Tris/HCl pH 7.2, ~15ml/ tube and pour into a sterile 250 ml beaker. Rinse tubes 2x's by vortexing and combine in beaker (note 7). Adjust the volume to 150 ml with Tris/HCL and then the final volume to 200 ml with buffered water.

14._____ Weigh out 20 mg of collagenase per 30 g starting tissue and dissolve in a small volume (~1 ml) of buffered water. Sterile filter through a 0.2 µm acrodisc syringe filter using a 1 cc syringe directly into the beaker containing the resuspended pellet. Add 60 µl 1 M calcium chloride into beaker to activate the enzyme. Cover and digest for 3 hrs at 37°C, while stirring.

15._____ Weigh out Trypsin and Chymotrypsin (20 mg of each per 30 g starting tissue) and dissolve in ~1 ml of buffered water. Sterile filter through a 0.2 µm acrodisc syringe filter using a 1 cc syringe directly into the beaker containing the resuspended pellet. Cover and digest for an additional 2 hrs at 37°C.

16._____ Pour supernatant into the 50 ml Oakridge centrifuge tubes as before, balance and place at 4°C for overnight storage.

Day 3

17._____ Centrifuge balanced tubes at 4°C for 10 min at 10,000 x g. Discard supernatant into biohazard waste.

18._____ During centrifugation set up the separatory funnel. Place funnel at a 45° angle with a sterile beaker underneath it to prevent leakage.

19._____ Shake 6% PEG/8% Dextran/9% Phosphate saline buffer to mix the two layers and resuspend the pellets in this buffer. Pour suspension into 400 ml beaker. Rinse tubes 2x by vortexing (note 7).

20._____ Adjust resuspension volume to 10 ml per gram of starting tissue with 6% PEG/8% Dextran/9% Phosphate saline buffer.

21._____ Mix by swirling beaker gently and pour into sterile separatory funnel. Allow mixture to separate for 1 hr at room temperature with minimal disturbance.

22._____ Slowly drain lower phase into beaker. Wait 5-10 min for layers to resettle.

23._____ Using a 25 or 50 ml sterile pipet (depending on volume), remove top phase and transfer to new sterile 50 ml Oakridge tubes ~25 ml per tube. This will be called the first upper.

24._____ Take the beaker where the funnel was drained and add 6% PEG/8% Dextran/9% Phosphate saline buffer to the same final volume as in step 20.

25._____ Repeat steps 21-23. This top layer removed at this step will be considered the second upper (note 8).

26._____ Place 20-25 ml of 0.2% Tween 80 solution in each tube, balance and centrifuge at 27,000 x g for 30 min at 4°C. Discard supernatant into biohazard waste.

27._____ Resuspend pellet in buffered water, combining all first upper pellets into one tube and all the second upper pellets into a different tube (note 7). Keep all the rinses with its corresponding upper tube, do not mix the rinse liquid with different upper tubes.

28._____ Balance and centrifuge tubes at 10,000 x g for 10min at 4°C. Discard supernatant into biohazard waste.

29._____ Resuspend pellet in buffered water and centrifuge balanced tubes at 10,000 x g for 10 min at 4°C. Discard supernatant into biohazard waste (note 9).

30._____ Resuspend pellets with 1-5 ml buffered water and transfer to a cyrotube. Rinse Oakridge tube and transfer to cryotube.

31._____ Perform a sterility test with a small amount of cell suspension in the cryotube as done in step 6.

32._____ Prepare a slide by adding 5 µl of each upper and spreading the sample evenly with the pipet tip and let the slide rest in the hood until dry. Perform an acid-fast stain and take a picture, making sure no other microorganisms are visible indicating contamination or other cellular debris (note 10).

33._____ Prepare 1:100 and 1:200 of sample: buffered water dilution in a total volume of 2 ml for each upper collected in glass test tubes and take an optical density reading at a wavelength of 540.

34._____ Record readings and use the formula (note 11) to calculate the concentration of cells in the cryovial. Note the final pellet color, pigment contamination and tissue contamination (note 12).

35._____ If there is no growth on TSA plate, BHI and LJ slant then these tubes can be used in other protocols.

Notes:

1. Liver and spleen samples are kept at -80°C to prevent degradation.
2. Glassware, tubes, stir bars, scissors and homogenizer chamber should be washed and autoclaved at 120°C for 50 min prior to use. Cover beakers with foil before autoclaving to ensure sterility.
3. See SOP: R015 M. leprae Buffers
4. A higher setting will create more bubbles and make it more difficult to work with. The top of the chamber might be warm or hot to the touch so care should be taken when removing the chamber from the homogenizer.
5. Incubate BHI media and TSA plates at 37°C for 4 days and the LJ slant at 37°C for 2 weeks.
   a. **BHI:** Check for turbidity
   b. **TSA:** Check growth and record growth characteristics

c. **LJ Slants:** Check for growth. Positive contamination is represented by orange/white colonies. Note: LJ slant may turn orange with age and may display a false positive.

6. Placing a large stir bar under the beaker while pouring will make it easier to pour the beaker contents without pouring the stir bar too.
7. When rinsing tubes, pour ~15 ml of buffer to one tube, vortex to insure that the pellet is resuspended and transfer to the next tube. Continue vortexing and transferring until all the tubes have been rinsed. One more rinse is done to make sure that particles from the pellet are not on the sides of the tube. If the pellet is difficult to resuspend more buffer maybe be used.
8. If the tissue size is small and/or if the 1st upper did not look very cloudy (indicating that there is low amount of cells) then taking a second upper is not necessary as it will give a very low yield with a higher tissue contamination. Continue with the other steps for the first upper.
9. This secondary wash is to make sure that the PEG/Dex is completely washed out of the cell pellet.
10. See SOP:SP035 Acid Fast Stain
11. Follow this formula for each upper.

    0.362 x _____(OD_{540}) x $100_{(dilution)}$ =_____mg/ml

    0.362 x _____(OD_{540}) x $200_{(dilution)}$ =_____mg/ml

    Average = _____mg/ml

    _____mg/ml x _____ml = _____mg
12. The final pellet color can range from white to dark brown. The pigment contamination scale is a number assigned indicating color #1= white/ cream to #5= dark brown. Only pigment contamination number 1-2 pass QC. Tissue contamination is assigned to each tube and is based on the slide and the presence of tissue particles. Tissue particles usually look like little blue spots if they're even visible. First upper is usually 1% tissue contamination, and 2nd upper is 5% unless the slide indicates a higher amount of tissue contamination.