Protocol: Isolation of Bacterial Membrane Vesicles

Protocol Notes:

- Grow pseudomonas from frozen stock to provide supernatant for membrane vesicles
- Purpose is to isolate the bacterial membrane vesicles using ultracentrifugation

Materials:

1. Pseudomonas aeruginosa ATCC #27853, Location: Freezer -80 #2, Shelf 4
2. Gentamicin stock 50 mg/ml, Location: Refrigerator #20
3. Mueller Hinton Broth (MHB), Location: Refrigerator #20 (prepared)
    - Sigma-Adlrich Mueller Hinton Broth, Cat#70192, Lot#
    - Store prepared media at 4 C in dark
    - Store dehydrated powder in dry location, Location:
4. Flask 250 mL, clear sterile plastic
5. Inoculating loops, sterile plastic, single use
    - Fisherbrand, Cat#13-075-1
6. 0.45 & 0.22 micron cellulose acetate membrane filters
    - Osmonics?
7. 50-mL polystyrene conical tubes (Falcon tube)
8. Beckman SW 41 Ti rotor, serial# 97U 10110 (swing-bucket rotor)
9. Beckman SW 41 Ti 104.9 buckets (6)
10. Beckman Spinkote, Cat# 306812
11. Beckman Vacuum Grease Silicone, Cat# 335148
12. Beckman Coulter Polyallomer Thin-walled Centrifuge Tubes, 14 x 89 mm, 13.2 mL capacity
    - Lot# Z11208SCA, Reorder# 331372
    - can sterilize acid or alkali, not alcohols

13. Sorval Legend XTR Centrifuge
14. Beckman Optima L-70k Ultracentrifuge
15. Ice
16. HEPES buffer 50 mM, pH 6.8
    - Sigma
17. NanoDrop ND-1000
18. (Micro) BCA protein assay reagent kit
    - Pierce
    - mirco optimized for 0.5-20 mcg protein ml^{-1}
19. Trypticase Soy Agar (TSA) plate, Location: Refrigerator #4/20, BD Cat#B11043
    1. Dissovle 20 g into 500 mL ddH2O. Mix by swirling, microwave 3 minutes (setting?), mix by swirling, repeat 3-4 times until powder completely dissolved. Autoclave at 121 degrees C for 15 minutes. (IMED autoclave settings: easy cycle, liquid, 15 minutes) At this time, turn waterbath on to 55 degree C.
    2. Remove from autoclave, mix solution by swirling to avoid clump/chunks. Place in 55 degree C waterbath, lay out dishes in hood (save bags), pipette 20-25 mL per plate (make sure to swirl solution around to avoid solidifying). Let dry 10-20 minutes, cover, flip, store in original bags at 4 degrees C.

Methods:

1. Plate frozen stock cultures of *Ps. aeruginosa* on trypticase soy agar (TSA) plates. Grow *Ps. aeruginosa* at 37 degrees C for approximately 24 hours.
2. Prepare and autoclave 1 liter of cation-adjusted Mueller-Hinton Broth medium (containing 1.23 mM calcium chloride dihydrate and 1.03 mM magnesium chloride hexahydrate).
3. Add 200 mL of Mueller-Hinton Broth to sterile 250-mL flasks
4. Using a sterile 10 mcl inoculation loop, scrape a 2 colonies of *Ps. aeruginosa* growth from agar plates (from Day 1) and transfer into 250-mL flask with MHB in it (Step 3). Grow broth cultures at 37 degree C for 6 h with gentle shaking (Incubator/Shaker name)

5. Add stock concentration gentamicin to 250/500-mL bacterial culture flasks to final concentration of 8 mcL/mL. Incubate gentamicin in the culture flask x30 minutes on orbital shaker at 37 degrees C.

    - 32 mcL for 200 mL culture volume
    - 16 mcL for 100 mL culture volume

6. Obtain *Ps. aeruginosa* cultures from the shaking incubator and remove a 1-ml aliquot from each 250/500-ml culture to check the respective optical densities. Late exponential phase *Ps. aeruginosa* is used ($\sim 10^8$ cfu ml^{-1}), usually at six hours of incubation. What is the target optical density?

7. Using a serological pipet, remove entire volume of supernatant required at one time, transferring to sterile 50-mL polystyrene conical tubes

    - Place volume in excess of 72 mL into 4 degrees C at once
    - Note that only 72 mL can be processed at one time in the SW 41 Ti rotor
    - Multiple passes may need to be done, store excess supernatant at 4 C until processed

8. Using a serological pipet, transfer culture supernatant to ultracentrifuge tubes. Balance tubes with cold 50 mM HEPES using a scale.

    1. Fill tubes leaving only a few millimeters of empty space.
    2. Tubes must be weighed within 0.1 – 0.2 g of one another

9. Spin samples in ultracentrifuge, 6,000 g (?? RPM with SW 41 Ti) for 30 minutes at 4 degrees C.

    1. This is saved as Program ? on the Optima L-70k Ultracentrifuge
    2. *Supernatant* after this step contains the membrane vesicles.
    3. Discard the pellet.

10. Filter supernatant sequentially through 0.45 and then 0.22 micron cellulose acetate filters

11. Using a serological pipet, transfer filtered supernatant to ultracentrifuge tubes. Balance tubes with cold 50 mM HEPES using a scale.

    1. Fill tubes leaving only a few millimeters of empty space.
    2. Tubes must be weighed within 0.1 – 0.2 g of one another

12. Spin samples in ultracentrifuge, 150,000 g (?? RPM with SW 41 Ti) for 90 minutes at 4 degrees C.

    1. This is saved as Program ? on the Optima L-70k Ultracentrifuge

2. *Pellet* after this step contains the membrane vesicles. Mark location of pellet with pen to facilitate extraction.
3. Discard the supernatant.

13. Again, use a serological pipet to carefully aspirate the supernatant from the samples. Use a pipetman to remove as much supernatant from the pellet as possible.

14. Resuspend membrane vesicle pellet in 50 mM HEPES buffer and using serological pipet transfer the suspension to ultracentrifuge tubes. Balance tubes with cold 50 mM HEPES using a scale.

    1. We do a two spin wash of the vesicle pellet
    2. Fill tubes leaving only a few millimeters of empty space.
    3. Tubes must be weighed within 0.1 – 0.2 g of one another

15. Spin samples in ultracentrifuge, 16,000 g (?? RPM with SW 41 Ti) for 30 minutes at 4 degrees C.

    1. This is saved as Program ? on the Optima L-70k Ultracentrifuge
    2. *Pellet* after this step contains the membrane vesicles.
    3. Discard the supernatant.
    4. Wash 1

16. Again, use a serological pipet to carefully aspirate the supernatant from the samples. Use a pipetman to remove as much supernatant from the pellet as possible.

17. Resuspend membrane vesicle pellet in 50 mM HEPES buffer and using serological pipet transfer the suspension to ultracentrifuge tubes. Balance tubes with cold 50 mM HEPES using a scale.

    1. Fill tubes leaving only a few millimeters of empty space.
    2. Tubes must be weighed within 0.1 – 0.2 g

18. Spin samples in ultracentrifuge, 16,000 g (?? RPM with SW 41 Ti) for 30 minutes at 4 degrees C.

    1. This is saved as Program ? on the Optima L-70k Ultracentrifuge
    2. *Pellet* after this step contains the membrane vesicles.
    3. Discard the supernatant.
    4. Wash 2

19. Determine membrane vesicle protein content using a (Micro) BCA protein assay reagent kit using the NanoDrop.

20. Plate approximately 1/10 of each final pellet (~50 mcg from each pellet) on TSA plate and incubate at 37 degrees C x48h to ensure no bacterial colonies form.

- If no colonies form, then membrane vesicle pellet can be considered sterile

21. Freeze membrane vesicles at -80 degree C in freezer #2, shelf #4.

References:

1. Renelli2004 - DNA-containing membrane vesicles of Pseudomonas aeruginosa PAO1 and their genetic transformation potential. (main protocol)

2. Ellis2010 - Naturally produced outer membrane vesicles from Pseudomonas aeruginosa elicit a potent innate immune response via combined sensing of both lipopolysaccharide and protein components. (SDS-PAGE for LPS)

3. Bauman2006 - Purification of outer membrane vesicles from Pseudomonas aeruginosa and their activation of an IL-8 response.

4. Kadurugamuwa1995 - Virulence factors are released from Pseudomonas aeruginosa in association with membrane vesicles during normal growth and exposure to gentamicin: a novel mechanism of enzyme secretion. (original isolation technique, gel)

5. Huntley, J.F., Robertson, G.T., Norgard, M.V. Method for the Isolation of Francisella tularensis Outer Membranes. J. Vis. Exp. (40), e2044, doi:10.3791/2044 (2010). (isolation of membrane proteins themselves)

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