Protocol: Isolation of Bacterial Membrane Vesicles

Protocol Notes:[1]

- Grow pseudomonas from frozen stock to provide supernatant for membrane vesicles
- Purpose is to isolate the bacterial membrane vesicles using ultracentrifugation
- This protocol is suitable for serum, BALF, and most other biological fluids.
- Threads of ultracentrifuge buckets must be coated with Beckman Spinkote, and the gaskets must be coated with Beckman Vacuum grease.
- Must refrigerate SW 41 Ti rotor buckets at 4 degrees C prior to running.

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:
    - Dissolve 21 g into 1 L ddH20.
    - Add 1.23 mM calcium chloride dihydrate and 1.03 mM magnesium chloride hexahydrate FIX ME
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 Type 45 Ti rotor, serial# 11U 4663 (fixed angle rotor, made in 2011)
9. Beckman Spinkote, Cat# 306812
10. Beckman Vacuum Grease Silicone, Cat# 335148
11. Beckman Coulter Centrifuge Tubes, Thinwall, Polypropylene, 94 mL, 38 x 102 mm
    - Lot# , Reorder# 345775

[1] Authors: Created by Patil Injean on 2014-06-30; Modified by ALG 2014-07

- can sterilize acid or alkali, not alcohols

12. Beckman Cap/Spacer Assembly, Aluminum, Tube, 38 mm dia
    - Cat# 330901
    - Requires special neoprene O-rings (see below)
13. Beckman O-Ring or Gasket, Aluminum, 30.0 mm ID x 37.0 mm OD
    - Cat# 346242
    - For use with the aluminum caps/spacers and the thinwall polyallomer/propylene tubes in 45 Ti rotor
14. Beckman Optima L-70k Ultracentrifuge
15. Sorval Legend XTR Centrifuge
16. Sorval rotor F13-14-50cy
17. New Brunswick Scientific Incubator Shaker G25
18. Ice
19. HEPES buffer 50 mM, pH 6.8, prepared on N/A by N/A, Location: refrigerator #? in 250-mL glass reagent bottle
    - Sigma
20. NanoDrop ND-1000
21. (Micro) BCA protein assay reagent kit
    - Pierce
    - mirco optimized for 0.5-20 mcg protein ml^{-1}
22. Trypticase Soy Agar (TSA) plate, Location: Refrigerator #20, BD Cat#B11043
    1. Dissovle 20 g into 500 mL ddH2O. Mix by swirling, microwave 3 minutes, 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 1500 mL of Mueller-Hinton Broth to sterile 3L glass Erlenmeyer 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 approximately 6 h with gentle shaking (New Brunswick Scientific Incubator Shaker G25)
5. 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 ($\sim 10^9$ cfu ml^{-1}), usually at approximately six hours of incubation.
    - Based on this cfu, the OD would be 0.900
6. Add stock concentration gentamicin to 3L 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
7. Using a serological pipet, remove entire volume of supernatant required at one time, transferring to sterile 50-mL polystyrene conical 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
8. Spin samples in Sorval Legend XTR, 6,000 g (6000 RPM with F13-14-50cy) for 30 minutes at 4 degrees C.
    1. This is saved as Program ? on the Sorval Legend XTR centrifuge
    2. *Supernatant* after this step contains the membrane vesicles.
    3. Discard the pellet.
9. Filter supernatant sequentially through 0.45 and then 0.22 micron cellulose acetate filters to remove any bacteria.
10. 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
11. Spin samples in ultracentrifuge, 150,000 g (44,000 RPM with 45 Ti) for 3 hours 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.
4. Place volume in excess of 72 mL into 4 degrees C at once
5. Note that only 72 mL can be processed at one time in the SW 41 Ti rotor
6. Multiple passes may need to be done to process all of the supernatant

12. 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.
13. Resuspend membrane vesicle pellet in 50 mM HEPES buffer and using serological pipet transfer the suspension to 50-mL conical tubes. Balance tubes with cold 50 mM HEPES using a scale.

    1. We do a one 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

14. Spin samples in ultracentrifuge, 120,000 g (39,000 RPM with 45 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.

15. Using serological pipet, resuspend pellet in 200-400 mcL of 50 mM HEPES
16. Determine membrane vesicle protein content using a (Micro) BCA protein assay reagent kit using the NanoDrop.
17. 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

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

References:

1. 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. (isolation protocol using all ultracentrifuge, gel)

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. Renelli2004 - DNA-containing membrane vesicles of Pseudomonas aeruginosa PAO1 and their genetic transformation potential. (isolation protocol using benchtop for washes, requires 16,500*g capabilities)

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)

6. Beckman Coulter Rotor Conversions weblink