SOP: PP006.4
Modified 02/27/17 by KE

Preparation of CFPs Using 2L Stirred Cell

Materials and Reagents:
1. Harvested CFP (see SOP PP003)
2. 90% Isopropyl alcohol
3. 70% Ethanol
4. 10 mM ammonium bicarbonate
5. Endotoxin-free MilliQ water
6. 2L Stirred Amicon Ultrafiltration Cell (Millipore Model 2000)
7. Amicon tubing
8. 5000 MWCO Ultrafiltration Membrane, 150mm diameter (Millipore, PLCC15005)
9. 20 liter Amicon reservoir
10. Compressed N_2 cylinder
11. Dialysis tank (optional)
12. Dialysis tubing (3,500 MWCO) (optional)
13. PES Filter unit (0.2 μm)
14. 25 ml Pipettes
15. 225 ml conical tubes
16. 150 ml plastic container
17. Sodium Azide (optional, note 1)
18. Wrench
19. Teflon Tape

Protocol: (note 2)

1._____ Obtain a clean 20 liter amicon reservoir, cover all openings with aluminum foil, and autoclave on a fast exhaust cycle.

2._____ Obtain a clean 2L stirred amicon ultrafiltration cell, and tubing (must have metal on both ends of tubing) for connection to the amicon reservoir

3._____ Rinse all tubing and components of the amicon ultrafiltration unit with 70% ethanol, and allow to air dry.

4._____ Equilibrate the 5000 MWCO amicon membrane in 90% isopropanol (36 mL 100% isopropanol, 4 mL MilliQ H_2O) for 10 minutes with the shiny side down, then in MilliQ water for 30 minutes.

5._____ Assemble the amicon ultrafiltration unit with the shiny side of the amicon membrane facing up (note 3; diagram 1)

6._____ Fill the 20 liter amicon reservoir with the harvested CFP, securely replace the lid on the reservoir, and connect the nitrogen line to the input port.

7._____ Connect one end of the amicon tubing to the output port of the amicon reservoir and the other end to the input port on the top of the amicon ultrafiltration unit (note 4).

8._____ Connect the plug from the top of the unit into the controller and turn on to stir.

9._____ Place the tubing connected to the output port on the base of the amicon ultrafiltration unit in a receptacle to collect the waste.

10._____ Check that the pressure release valves on both the reservoir and the ultrafiltration unit are closed.

11._____ Turn on the nitrogen gas (note 5).

12._____ Check the amicon ultrafiltration unit to ensure the stirrer is turning, and that there are no leaks around the lid, base, or tubing connections. Also check to see that the CFP ultrafiltrate is slowly flowing from the outlet port at the base of the ultrafiltration unit (note 6).

13._____ Check the amicon ultrafiltration unit and reservoir daily and discard ultrafiltrate as needed (note 7).

14._____ When the volume of CFP in the amicon ultrafiltration unit is reduced to ~100 ml turn off the nitrogen gas and stirrer, and vent the system at both the reservoir and the stirred cell.

15._____ Add 4 L of 10 mM ammonium bicarbonate (at least 20 times the CFP volume) to the reservoir to begin buffer exchange (note 8).

16._____ Close all pressure release valves, turn on stirrer and nitrogen.

17._____ When the CFP in the amicon ultrafiltration unit is reduced to ~100 ml, shut down as previously described.

18._____ Unplug the unit, disconnect all tubing, and remove the lid from the ultrafiltration unit.

19._____ Transfer the concentrated filtrate to a 0.2 μm filter unit.

20._____ Wash the amicon membrane and ultrafiltration unit with ~ 15 ml of 10 mM ammonium bicarbonate and add the wash to the concentrated filtrate.

21._____ Filter sterilize the CFP.

22._____ Perform QC procedures as described below.

23._____ Store the CFP at -80°C, or lyophilize (see SOP SP004).

QC Procedures for H37Rv, EAI, HN878, and CDC1551 strains:

1. BCA assay (see SOP SP003)
2. SDS-PAGE (see SOP SP007)
3. Western Blot using the antibodies SA-12 (anti-GroES), CS-18 (anti-Rv3846), CS-90 (anti-Ag85), IT-23 (anti-PhoS1), CS-44 (anti-GroEL2 *negative control), IT-41 (anti-DnaK) (see SOP SP0011)
4. LAL assay for endotoxin (optional)
5. Default aliquot: 1 mg

QC Procedures for IO strain:

1. BCA assay (see SOP SP003)
2. SDS-PAGE (see SOP SP007)
3. Western Blot using the antibodies SA-12 (anti-GroES), CS-18 (anti-Rv3846), CS-90 (anti-Ag85), IT-23 (anti-PhoS1), IT-41 (anti-DnaK) (see SOP SP0011)
4. LDH Assay (see SOP SP049)
5. LAL assay for endotoxin (optional)
6. Default aliquot: 1 mg

QC Procedures for BAA strain:

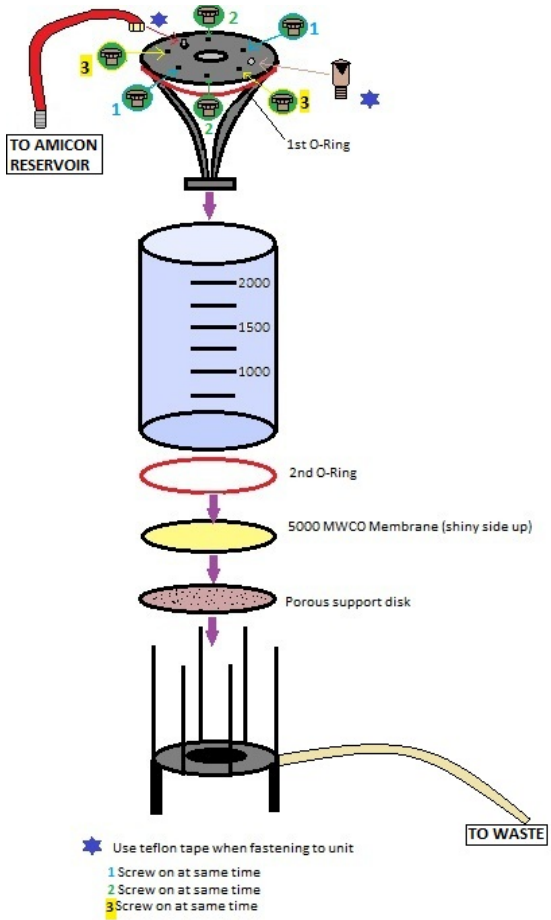
1. BCA assay (see SOP SP003)
2. SDS-PAGE (see SOP SP007)
3. Western Blot using the antibodies SA-12 (anti-GroES), CS-49 (anti-HspX *negative control), CS-93 (anti-Mpt32), IT-41 (anti-DnaK) (see SOP SP0011)
4. LAL assay for endotoxin (optional)
5. Default aliquot: 1 mg

Notes:

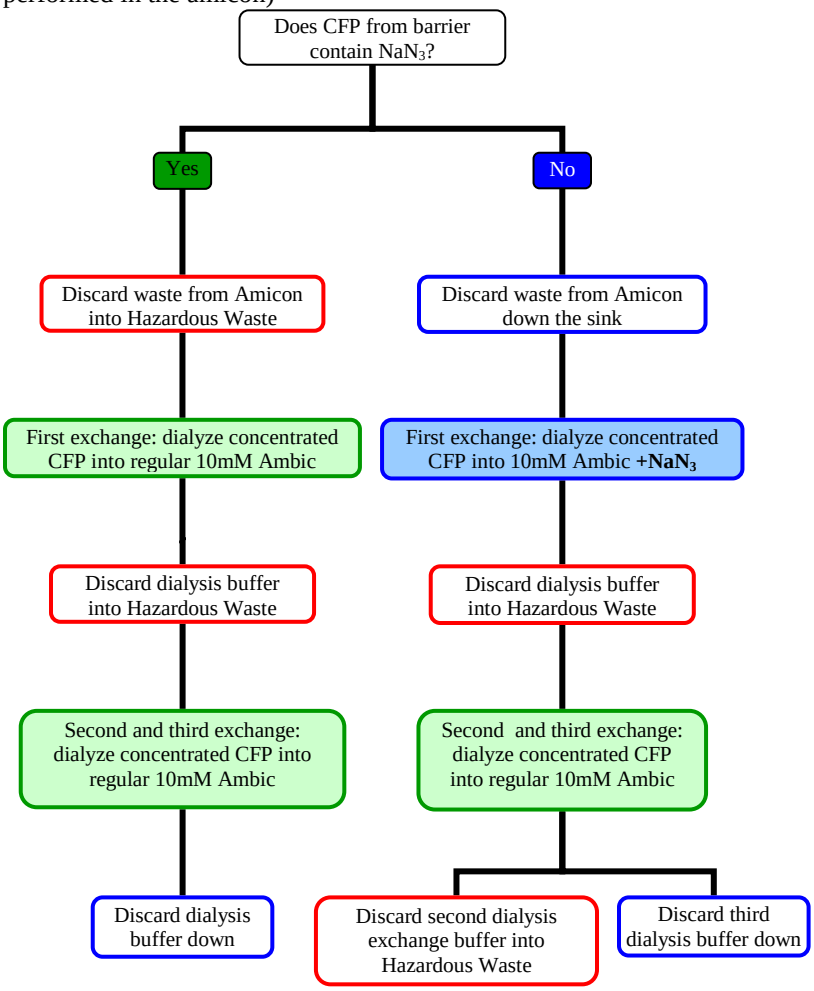
1. If there is a delay before the CFP can be processed, ~1g should be added to each 5L bottle of harvested CFP in order to prevent contamination. If sodium azide is used, the flow-through from the amicon and the buffer exchange (or dialysis buffers) must be disposed of as hazardous waste.
2. Amicon ultrafiltration must be performed at 4°C.
3. It is important that all O-rings are properly seated to prevent leakage.
4. Failure to properly connect and setup the amicon ultrafiltration unit will result in significant loss of CFP and the operator being sprayed once pressure is applied to the amicon unit.
5. The nitrogen pressure should read no greater than 60 psi on the regulator.
6. If a leak is found or the output flow is too great, shut off the flow of nitrogen, release pressure from the reservoir first, then from the stirred cell. Leaks from the lid are generally caused by a poorly seated o-ring. Leaks from tubing connections may require tightening or the use of Teflon tape. If the output flow is too fast, this could indicate a poorly seated membrane or o-ring, or a flaw in the membrane. Once the problem is corrected, start at step 7 and repeat startup procedures.
7. It generally takes 2 days to concentrate one 16 liter batch of CFP.
8. Alternatively, the CFP can be collected from the amicon prior to buffer exchange and subjected to traditional dialysis using 3500 MWCO dialysis tubing, three exchanges of 10 mM ammonium bicarbonate over a minimum of 24 hours. Refer to Diagram 2 for explanation handling of dialysis buffers containing sodium azide. After dialysis, resume protocol starting at step 21.

Diagrams:

1. Setup of amicon



2. Handling of dialysis buffers containing sodium azide (not applicable for samples where buffer exchange is performed in the amicon)



References:

Sonnenberg, M. G., and J. T. Belisle. 1997. Definition of *Mycobacterium tuberculosis* culture filtrate proteins by two-dimensional polyacrylamide gel electrophoresis, N-terminal amino acid sequencing, and electrospray mass spectrometry. Infect Immun 65:4515-24.