

**SOP: PP006.1**  
**Modified 1-22-07**

### **Preparation of CFPs Using 2L Stirred Cell**

**Materials and Reagents:**

1. Harvested CFP from *M. tuberculosis* (see SOP PP003)
2. 90% Isopropyl alcohol
3. 70% Ethanol
4. Dialysis buffer (10 mM Ammonium bicarbonate, 1 mM DTT, 0.02% NaN<sub>3</sub>)
5. 10 mM ammonium bicarbonate
6. Endotoxin-free MilliQ water
7. 2L Stirred Amicon Ultrafiltration Cell (Millipore Model 2000)
8. Amicon tubing
9. 5000 MWCO Ultrafiltration Membrane, 150mm diameter (Millipore, PLCC15005)
10. 20 liter Amicon reservoir
11. Compressed N<sub>2</sub> cylinder
12. Dialysis tank
13. Dialysis tubing (3,500 MWCO)
14. ZapCaps (0.2 µm)
15. 25 ml Pipettes
16. 225 ml conical tubes
17. 150 ml plastic container

**Protocol:** (note 1)

1. \_\_\_\_ Obtain a clean 20 liter amicon reservoir, cover all openings with aluminum foil, and autoclave on a fast exhaust cycle.
2. \_\_\_\_ Rinse all tubing and components of the amicon ultrafiltration unit with 70% ethanol, and allow to air dry.
3. \_\_\_\_ Obtain a clean 2L stirred amicon ultrafiltration cell, and tubing for connection to the amicon reservoir
4. \_\_\_\_ Equilibrate the 5000 MWCO amicon membrane in 90% isopropanol for 10 minutes with the shiny side down, then in MilliQ water for 30 minutes.
5. \_\_\_\_ Assemble the amicon ultrafiltration unit with the amicon membrane (note 2).
6. \_\_\_\_ Fill the 20 liter amicon reservoir with the *M. tuberculosis* 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 3).
8. \_\_\_\_ Connect the plug from the top of the unit into the controller and turn on to stir.
9. \_\_\_\_ Ensure the tubing connected to the output port on the base of the amicon ultrafiltration unit is placed in a receptacle to collect the ultrafiltrate.
10. \_\_\_\_ Turn on the nitrogen gas (note 4).
11. \_\_\_\_ Check the amicon ultrafiltration unit to ensure the stirrer is turning, and that there is no leakage 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 5).

12. \_\_\_\_ Check the amicon ultrafiltration unit and reservoir daily and discard ultrafiltrate as needed (note 6).
13. \_\_\_\_ When the volume of CFP in the amicon ultrafiltration unit is reduced to ~50 ml turn off the nitrogen gas, and vent the system at both the reservoir and the stirred cell.
14. \_\_\_\_ Turn off the stirrer and unplug the unit, disconnect all tubing, and remove the lid from the ultrafiltration unit.
15. \_\_\_\_ Transfer the concentrated filtrate to a 225 ml conical bottle.
16. \_\_\_\_ Wash the membrane and ultrafiltration unit with ~ 15 ml of 10 mM ammonium bicarbonate and add the wash to the concentrated filtrate.
17. \_\_\_\_ Prepare 3,500 MWCO dialysis tubing by boiling in endotoxin-free MilliQ H<sub>2</sub>O.
18. \_\_\_\_ Prepare 7 L of dialysis buffer in a dialysis tank.
19. \_\_\_\_ Add the concentrated CFP to the dialysis tubing. Close the dialysis tubing and place in the dialysis buffer.
20. \_\_\_\_ Dialyze at 4°C for 4 to 12 hours.
21. \_\_\_\_ Change the dialysis buffer (7 L) and dialyze at 4°C for 4 to 12 hours.
22. \_\_\_\_ Change the dialysis buffer to 7 L of 10 mM ammonium bicarbonate and dialyze at 4°C for 4 to 12 hours.
23. \_\_\_\_ Collect the protein solution from the dialysis tubing and rinse the dialysis tubing with a minimal volume (~5 ml) of fresh 10 mM ammonium bicarbonate.
24. \_\_\_\_ Filter sterilize the dialyzed CFP using a 0.2 µm ZapCap and collect in a sterile 150 ml plastic container.
25. \_\_\_\_ Perform QC procedures.
26. \_\_\_\_ Store the CFP at -80°C, or lyophilize (see SOP SP004).

**QC Procedures:**

1. SDS-PAGE (see SOP SP007)
2. Western Blot using the antibodies SA12, CS18, IT49, IT23, CS44, IT41 (see SOP SP0011)
3. BCA assay (see SOP SP003)
4. LAL assay for endotoxin (optional)
5. Default aliquot: 1mg

**Notes:**

1. Amicon ultrafiltration must be performed at 4°C.
2. It is important that all O-rings are properly seated to prevent leakage. Also the membrane should be placed in the base of the ultrafiltration unit so that the shiny side of the membrane is facing up.
3. 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.
4. The nitrogen pressure should read no greater than 60 psi on the regulator.
5. If a leak is found or the output flow is too great, shut off the flow of nitrogen, release pressure from the reservoir, and correct the problem. Then start at step 7 and repeat startup procedures.

6. It generally takes 2 days to concentrate one 21 liter batch of CFP.

**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.