

SOP: PP005

Preparation of CFPs and Low MW CFPs

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% NaN₃)
5. 10 mM ammonium bicarbonate
6. Endotoxin-free MilliQ water
7. 350 ml Amicon ultrafiltration unit
8. 10,000 MWCO membrane (Millipore catalog number PLGC07610) or YM3 membrane (Millipore catalog number 13442AM) (note 1)
9. Amicon tubing (2)
10. Amicon ultrafiltration unit housing
11. 10 liter Amicon reservoir
12. Compressed N₂ cylinder
13. Magnetic stir plate
14. Dialysis tank
15. Dialysis tubing (3,500 MWCO)
16. ZapCaps (0.2 µm)
17. 25 ml Pipettes
18. 225 ml conical tube
19. 150 ml plastic container

Protocol: (note 2)

1. ____ Obtain a clean 10 liter amicon reservoir, cover all openings with aluminum foil, and autoclave on a fast exhaust cycle.
2. ____ Obtain a clean 350 ml amicon ultrafiltration unit, and 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 10k MWCO (or YM3) amicon membrane, with the shiny side down, in 90% isopropanol for 10 minutes, then in MilliQ water for 30 minutes.
5. ____ Assemble the amicon ultrafiltration unit with the 10k MWCO (or YM3) amicon membrane (note 3).
6. ____ Fill the 10 liter amicon reservoir with the *M. tuberculosis* CFP, securely replace the lid to the reservoir, and connect the nitrogen line to the input port.
7. ____ Connect one end of amicon tubing to the output port of the amicon reservoir and the other end to the input port on the top (lid) of the amicon ultrafiltration unit.
8. ____ Place the amicon ultrafiltration unit in the housing and close the vent on the back of the lid.
9. ____ Place the amicon ultrafiltration unit and housing on a magnetic stir plate and turn the stir plate on.
10. ____ 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.
11. ____ Turn on the nitrogen gas (note 4).

12. ____ 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).
13. ____ Check the amicon ultrafiltration unit and reservoir daily and discard ultrafiltrate as needed (note 6).
14. ____ When the CFP in the reservoir becomes low or empty turn off the nitrogen and release pressure by opening the vent valve on the top of the reservoir. If required add more CFP of the same lot to the reservoir, close the vent valve and repeat steps 11 to 13. Repeat until all the CFP is concentrated.
15. ____ When the volume of CFP in the amicon ultrafiltration unit is reduced to ~50 ml turn off the nitrogen gas, and vent the system.
16. ____ Remove the amicon ultrafiltration unit from its housing, disconnect all tubing, and remove the lid of the ultrafiltration unit.
17. ____ Transfer the concentrated filtrate to a 225 ml conical bottle.
18. ____ Wash the membrane and ultrafiltration unit two times with ~ 10 ml of 10 mM ammonium bicarbonate and add the washes to the concentrated filtrate.
19. ____ Prepare 3,500 MWCO dialysis tubing by boiling in endotoxin-free MilliQ H₂O.
20. ____ Prepare 7 L of dialysis buffer in a dialysis tank.
21. ____ Add the concentrated CFP to the dialysis tubing. Close the dialysis tubing and place in the dialysis buffer.
22. ____ Dialyze at 4°C for 4 to 12 hours.
23. ____ Change the dialysis buffer (7 L) and dialyze at 4°C for 4 to 12 hours.
24. ____ Change the dialysis buffer to 7 L of 10 mM ammonium bicarbonate and dialyze at 4°C for 4 to 12 hours.
25. ____ 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.
26. ____ Filter sterilize the dialyzed CFP using a 0.2 µm ZapCap and collect in a sterile 150 ml plastic container.
27. ____ Estimate protein concentration using a BCA assay (see SOP SP003).
28. ____ Run 4-5 µg of protein on a QC gel (see SOP SP007) and blot (see SOP SP011). Develop the blot using the QC antibodies SA12, CS18, IT49, IT23, CS44, IT41, or for Low MW CFP, use α-CFP10, α-ESAT6, SA12, IT49, IT23.
29. ____ Aliquot the CFP (default aliquot = 1 mg) and store at -80°C, or lyophilize (see SOP SP004).

Notes:

1. For low MW CFP, use the YM3 membrane and only use 2-4L of crude CFP for the starting material.

2. Amicon ultrafiltration must be performed at 4°C.
3. 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 10 to 14 days to concentrate one 21 liter batch of CFP. The reservoir will empty in about 2-3 days depending on the concentration of the 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.