**SOP: PP056**
**Created 6/25/20 MCL**

**Mycobacterial Extracellular Vesicle (MEV) Enriched and Depleted CFP**

**Materials and Reagents:**

1. Concentrated Culture Filtrate Proteins (CFP) from *Mycobacterium spp* (*see* **SOP: PP006**, *see* **Note 1**)
2. 100 kDa MWCO centrifugal filter
3. 3 kDa MWCO centrifugal filter
4. 10 mM ammonium bicarbonate
5. Capto™ Core 700 multimodal chromatography resin
6. Disposable gravity flow column with cap
7. Leur-lock syringe
8. 0.22 µm polyethersulfone (PES) syringe filter
9. Bicinchoninic acid (BCA) protein assay kit (*see* **Note 2**)
10. microBCA protein assay kit (*see* **Note 2**)
11. SDS-PAGE gel
12. Nanoparticle Tracking Analysis (NTA) instrumentation (*see* **Note 3**)

**Protocol:**

1._____ Thaw CFP in a 4 °C refrigerator overnight (*see* **Note 4**).

2._____ Rinse a 100 kDa centrifugal filter with 10 mM ammonium bicarbonate: add 10 mM ammonium bicarbonate to the unit capacity. Spin the device at its maximum centrifugal force for 5 min and discard any remaining retentate as well as the eluate.

3._____ Fill the 100 kDa centrifugal filter with thawed CFP from **step 1** (retain a small aliquot of CFP for comparison to final product during QC). Spin the device at its maximum centrifugal force at 4 °C until the sample has reduced to the desired volume. The eluate is MEV depleted CFP. Retain the eluate and store at 4 °C for downstream analysis.

4._____ Perform wash on retentate: add 10 mM ammonium bicarbonate to the sample in the 100 kDa centrifugal filter and centrifuge as in **step 3** until the sample has reduced to the desired volume. Save the eluate in a clean tube and repeat the wash with addition of 10 mM ammonium bicarbonate for a total of at least three times, pooling the eluate from each buffer exchange cycle.

5._____ Recover the retentate in a clean tube.

6._____ Gently rinse the 100 kDa centrifugal filter membrane with 10 mM ammonium bicarbonate, and add this to the saved retentate.

7._____ Perform a BCA on the eluate and retentate to determine protein concentration (*see* **Note 5)**.

8._____ Store the eluate and an aliquot of the retentate at 4 °C for QC.

9._____ Dilute 10 mg of 100 kDa retentate from **step 6** to a volume of 5 mL with 10 mM ammonium bicarbonate, mixing gently.

10._____ Add 1 mL of well-mixed Capto™ Core 700 multimodal chromatography resin to an empty gravity flow column. Place the column in a size appropriate rack fitted with a drip tray.

11._____ Add 10 mL of 10 mM ammonium bicarbonate to wash out the storage solution from the resin. Allow 5 min for the wash to drain from the column. Cap the bottom of the column after draining is complete.

12._____ Gently transfer the 10 mg of diluted 100 kDa retentate from **step 9** to the column (*see* **Note 6**); take care not to cause excess disruption of the resin bed. Allow the sample and resin to settle for 5 min.

13._____ Place a clean collection tube under the gravity flow column, remove the cap, and allow the sample to flow through the resin.

14._____ Cap the gravity flow column and apply the collected material to the resin again to remove any remaining material below 700 kDa. Allow the sample and resin to settle for 5 min.

15._____ Place a new collection tube under the gravity flow column, remove the cap, and allow the sample to flow through the resin.

16._____ Leaving the cap open, add 1 mL of 10 mM ammonium bicarbonate and collect the wash in the tube from **step 15**.

17._____ Repeat **step 16**. The total final volume will be roughly 7 mL.

18._____ Rinse a 3 kDa centrifugal filter with 10 mM ammonium bicarbonate prior to use, as described in **step 2.**

19._____ Add the sample from **step 17** to the 3 kDa centrifugal filter. Spin the filter at its maximum centrifugal force at 4 °C until the sample has reduced to the desired volume.

20._____ Transfer the retentate to a new tube.

21._____ Rinse the filter membrane with 200 µL 10 mM ammonium bicarbonate and add the rinse to the retentate sample. The eluate can be discarded.

22._____ Filter the vesicle sample using a leur-lock syringe and 0.22 µm PES syringe filter into a new, sterile tube. Keep the sample at 4 °C for short-term storage.

23._____ Quantify the protein content of the sample by BCA, following the manufacturer's protocol (*see* **Note 5)**.

24._____ Take 5 µg each of the original CFP sample (*see* **step 3**), the 100 kDa eluate (*see* **step 8**), and the purified vesicle sample (*see* **step 22**) for SDS-polyacrylamide gel electrophoresis.

25._____ Visualize the proteins by silver staining (*see* **SOP:** SP012) or Coomassie® Blue Staining following standard recommendations to evaluate soluble protein reduction.

26._____ Perform western blot to confirm the presence or absence of specific proteins (*see* **Note 7**).

27._____ Dilute 5 µg of the vesicle sample (as quantified in **step 23**) to 1 mL using 10 mM ammonium bicarbonate; vortex for 15 sec on medium speed.

28._____ Draw the sample into a 1 mL disposable syringe. If available, set the syringe in an automatic syringe pump set to inject the diluted sample at a rate of 30 µL/min.

29._____ Visualize the sample, adjusting the screen gain, cameral level, and focus as needed. Consult the instrument user manual for guidance regarding set up and optimization (*see* **Note 8**).

30._____ Capture video of three technical replicates for a minimum of 30 sec, using a constant flow for every replicate. Adjust the capture threshold for analysis; consult the instrument user manual for guidance (*see* **Note 9**).

31._____ Make aliquots in 1.2 ml cryovials:
MEV Enriched CFP: 0.05 mg/vial
MEV Depleted CFP: 0.25 mg/vial

32._____ Store at -80 °C.

**Notes:**

1. Other size exclusion chromatography resins can be used. However, traditional resins lack the binding properties and resulting dual functionality of Capto™ Core 700 and non-MEV proteins will eventually elute. The vesicle enrichment described in this protocol is specific to this multimodal resin.
2. Alternative ultra-sensitive protein quantitation method may be used as long as it is compatible with the buffers used. Be sure to use protein standards made in the same buffer as your sample.
3. Alternative vesicle quantification and sizing techniques can be applied; these include but are not limited to: Dynamic Light Scattering (DLS) and Tunable Resistive Pulse Sensing (TRPS).
4. The starting product can be transitioned directly upon completion of the culture filtrate protocol (**SOP: PP006**) prior to freezing, eliminating the need for thawing.
5. If sample is too dilute, microBCA may be used for quantitation.
6. The maximum protein binding capacity for Capto™ Core 700 is 14 mg/mL of resin.
7. Suggested antibodies for western blot analysis (additional/different antibodies may be used as markers are optimized):
   <u>For EV enriched CFP</u>
   - Positive control: anti-GlcB (Rv1837c), anti-LpqH (Rv3763)
   - Negative control: anti-GroES (Rv3418c)

   <u>For EV depleted CFP</u>
   - Positive control: anti-GroES (Rv3418c)
   - Negative control: anti-LAM (anti-LpqH or anti-GlcB may also be used as negative control)
8. If the sample is too concentrated for accurate analysis, perform 1:10 serial dilutions from the 5 µg sample in 1 mL 10 mM ammonium bicarbonate until an appropriate concentration is achieved. In contrast, if the sample is too dilute, an additional 100 kDa concentration may be performed (*see* **steps 2 through 6**), followed by microBCA assay.
9. The capture and analysis settings for NTA must be consistent across samples for comparability. Alternatively, TEM can also be performed to visualize size and vesicle integrity