



Protocol #5: Processing of culture filtrate for secreted proteome analysis by size fractionation for high and low molecular weight protein/peptide analysis

Purpose:

This protocol describes steps to fractionate proteins/peptides from Mtb cell culture filtrate into 1) a high molecular weight proteome fraction, **F1**; 2) a middle molecular peptidome fraction, **F2** and 3) a low molecular weight peptide and small molecular fraction, **F3** using a combination of 3K (3,000) Nominal Molecular Weight Limit (NMWL) and 30K (30,000) filter devices.

Materials:

Mtb cell culture filtrate

Centrifugation devices for size fractionation and concentration: 30K filter device (Millipore® Amicon Ultra-15, 30K NMWL, cat # UFC903096) and 3K filter device (Millipore® Amicon Ultra-15, 3K NMWL, cat # UFC900396)

Eppendorf microcentrifuge 1.5mL, low protein binding (VWR, cat # 80077-232)

Eppendorf microcentrifuge 2.0mL, low protein binding (VWR, cat # 80077-226)

Process:

1. Remove Mtb cell culture filtrate sample (~ 50 mL) from -80 °C freezer and thawed at room temperature in a water bath for 1.5 hours.
2. Pre-rinse and leak check 30KD and 3KD filter devices each with 15 mL HPLC water.
3. Add 10mL of the thawed sample to the pre-rinsed and labeled 30kD filter unit.
4. Centrifuge the capped 30kD sample filter device at 3220g (4000rpm) x 45 mins, 25 °C.
5. After centrifugation, transfer the retentate in the filter unit to a 2.0mL low protein binding Eppendorf tube labeled as **F1**, a high molecular weight proteome fraction. Vortex **F1** sample from step 5, measure and record sample volume (~ 200 uL). Process the sample subsequently following Protocol #1 with 2 doses of trypsin (2 µg each) for digestion.
6. Transfer filtrate (flow-through) in the 30KD filter device into a filter unit of the pre-rinsed and labeled 3 kD filter unit.
7. Centrifuge the capped 3KD sample device at 3220g (4000rpm) x 60 mins, 25 °C.
8. After centrifugation, transfer filtrate (flow-through) to a 15mL Falcon tube, labeled as a low molecular weight peptide fraction (**F3**), for subsequent processing for removing salt and detergent molecules.
9. Add 12 mL HPLC water to retentate in the 3KD filter unit, close cap and mix well.
10. Centrifuge the capped 3KD sample device at 3220g (4000rpm) x 70 mins, 25 °C.
11. After centrifugation, discard the filtrate and transfer the retentate in the filter unit to a 2.0mL low protein binding Eppendorf tube labeled as **F2**, a middle molecular peptidome fraction for direct LC-MS/MS analysis.

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