PURIFICATION OF PROTEINS FROM PFA FIXED SAMPLES BAK 2023

"Extraction of Proteins from Formalin Fixed Tissues"

Wash Buffer (1L)

1. 100mL 10X TBS pH 7.6.
2. 10mL Triton X-100.
3. 1g SDS.
4. 2 mL 0.5M EDTA pH 8.0.

Dissolve in milliq water. Up to final volume 1L. Store at room temperature for 6 months.

Reversal Buffer (RB) (500ML)

1. 25g SDS.
2. 2 mL of 0.5M EDTA pH 8.0.
3. 30.35 g of Tris-Base.
4. 4.38 g of NaCl.

Dissolve in milliq water. pH to 7.6. Up to final volume 500mL. Store at room temperature for up to 1 year.

PMSF solution (100 mM). Acute toxicity, handle powder carefully.

1. Place 1.5mL tube onto scale, tare.
2. Add small amount of PMSF to tube, record weight.
3. Add calculated volume of isopropanol to PMSF. Mix well. To calculate: PMSF mass (mg) / 17.4 = volume of isopropanol.

Stable in isopropanol at RT for at least 6 months. Stable for ~15 min once added to aqueous solution.

Procedure Day 1 (Extract proteins and test concentration)

a. Wash sections in DM 3 X 10 min.
b. Place sections in 1.5mL Eppendorf tube.
c. Add 0.5mL of reversal buffer.
d. Briefly sonicate on low power to disperse tissue.
e. Add 5µl of 100mM PMSF. Mix well. Quick spin.
f. Heat on block for 98°C for 30 min with cap locks
g. Remove from heat block carefully (caps will pop if not careful).
h. After 5 min of cooling, vortex well.
i. Centrifuge at 22,000 x g for 30 min RT.
j. Collect S1 (extracted proteins).

2. Western blot (Optional)
    a. Perform methanol/chloroform cleanup on 100ul of S1.
    b. Resuspend resulting pellet in 100ul 5% SDS.
    c. Perform BCA assay on 100ul. Each BCA test takes 20ul. The remaining volume can be used for western blot. Alternatively, more protein can be prepared from S1 if needed.
    d. Calculate volume required for 20µg protein.

i. 20ug / concentration of S1 ug/ul = ul of sample required

Procedure Day 2 (Capture biotinylated proteins and wash)

1. Add S1 to 10mL TBST in 15mL conical tube. Mix well.
2. Add 40 ul of prepared magnetic streptavidin beads to each tube.
3. Nutation for 1h RT.
4. Place tube on magnetic stand for 1 min (until all beads have been drawn out of solution).
5. Carefully remove supernatant no to disturb beads. Add 10mL wash buffer.
6. Nutation for 30 min.
7. Place tube on magnetic stand for 1 min (until all beads have been drawn out of solution).
8. Add 10mL wash buffer.
9. Nutation for 30 min.
10. Place tube on magnetic stand for 1 min (until all beads have been drawn out of solution).
11. Add 10mL wash buffer.
12. Nutation overnight at 4°C.

Day 3 (Elute captured proteins)

1. Place tube on magnetic stand for 1 min (until all beads have been drawn out of solution).
2. Carefully remove supernatant no to disturb beads.
3. Add 1mL of wash buffer.
4. Mix samples until beads are suspended in wash buffer.
5. Using a pipette with tip cut off, transfer suspended beads to a 1.5mL protein low bind tube.
6. Place tube on magnetic stand for 1 min (until all beads have been drawn out of solution).
7. Remove buffer and surface wash with milliq 2X.
8. Quick spin and remove liquid from tube (should only have beads left).
9. Add 80µl 1X SDS-page sample buffer containing reducing agent.
10. Mix well and quick spin.
11. Place on heat block set to 98°C for 10 min.
12. Mix well and quick spin.
13. Place on magnet.
14. Transfer eluent to two separate lo-bind tube and discard used beads. 35 ul in 1 tube (used for premass spec QC), the remaining 45 ul in another (used for mass spec).
15. Immediately place in -80°C for long-term storage.

Day 4 (Prepare proteins for Mass Spec)

1. Prepare Bis-Tris 4-12% wedge gel 15 well.
2. Make 800 mL 1X MOPS running buffer.
3. Load 5ul of MW standard in one side of gel.
4. For MS load exactly 40 ul of the samples into wells. Careful not to spill into opposing wells. Only load samples into every other well to prevent cross contamination.
5. Ensure all wells have a buffer. For empty wells fill with 40ul 1X loading buffer.
6. Run the gel at 150V for a few minutes. Watch carefully until the sample has completely entered gel.
7. Stop running, remove gel with clean gloves, and clean equipment.

8. Fix gel in 100mL fixation solution (50% Ethanol, 10% acetic acid) for 1hr RT.
9. Wash gel in several changes of milliq, until gel has swollen to original size.
10. Place in 100mL colloidal Coomassie blue stain solution. Cover loosely, and heat in microwave for few minutes until solution just starts to boil.
11. Incubate gel in heated colloidal Coomassie blue stain solution for 8 min.
12. Remove gel from stain solution and wash several times with milliq water. Clear background is achieved with ~2h of washing.
13. Using a clean razor excise the entire sample from the gel.
14. Place each sample into a clean 1.5mL tube. Add 500ul of milliq water.
15. Samples can be stored at 4°C until submitted to mass spec core.

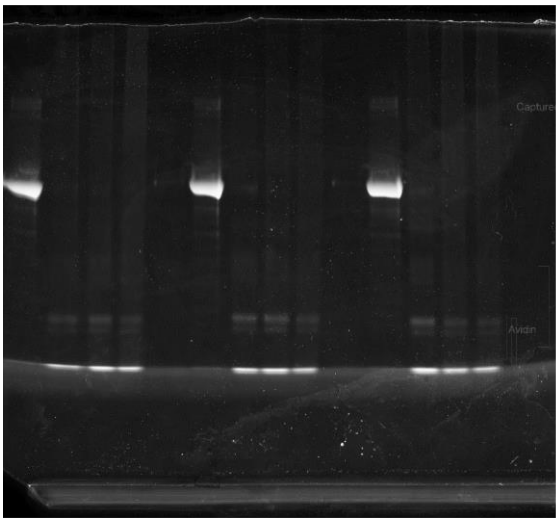
Optional: Pre mass spec QC

Estimating capture concentration

Purpose: To provide mass spec core with a rough estimate of the amount of protein in the capture sample. This prevents potential overloading, or digestion problems in the mass spec core.

1. Prepare gel as described above (Day 4 Steps 1-3)
2. Load 20ul of QC aliquot into wells.
3. Load 2ug BSA into 1 well.
4. Run the gel at 150V for approximately 45min or until the die has reached end of gel.
5. Stain gel with Coomassie as described above (Day 4 Steps 8-12)
6. Image on Odyssey using NIR.
7. Forward image to NW mass spectrometry core.

Example of results; order = 2ug BSA, 3 captures, 2ug BSA, 3 captures, 2ug BSA, 3 captures



Dot blot to estimate target enrichment

1. Cut PVDF membrane into ~1X3 inch piece.
2. Activate PVDF in 100% methanol for 30s.
3. Equilibrate activated PVDF in diH20.
4. Just prior to blotting samples, place hydrated PVDF on wypall and quickly pat dry.

5. Place PVDF on new dry wypall.
6. Spot 1ul of each sample onto PVDF
7. Allow PVDF to dry completely. (~1h or overnight)
8. Reactivate spotted PVDF as described above and equilibrate in dH20.
9. Blot can now be probed with antibodies using standard protocols.

Example of results