Yan Lab – Patrick Fubio 8/18/2026

Isolation of Mitochondria from Tissue via Differential Centrifugation v.5

This protocol is for accurate measure of phosphoprotein expression in mitochondria fractions from tissues in mice via Western blot. Based on a protocol from Reisman et al (bioRxiv, 2026).

Procedures

1. Pre-cool Eppendorf 5417R Refrigerated Benchtop Centrifuge to 4°C. Keep 1X TBS, 2X SB, and MIB with inhibitors on ice.
2. Dissect tissue(s) of interest. If necessary, quickly rinse harvested tissue in ice cold 1X PBS to remove blood and weigh. If rinsed in 1X PBS, blot dry with KimWipe before mincing.
3. Mince tissue quickly for 10 seconds with scissors on parafilm.
4. Transfer minced tissue to 5 mL Falcon Round Bottom tube on ice.
5. Add approximately 20x volume (v/w) of ice-cold in MIB with inhibitors immediately after mincing. For liver, add approximately 25x volume.
6. Homogenize the tissue in 5 mL tube placed in beaker with ice water in ice bucket. Perform three 10-sec pulses with Polytron hand homogenizer at 60% of maximum speed, resting 5-10 seconds in between pulses. Avoid bubbles and ensure sample is completely homogenized. Rinse Polytron by running in ice-cold DI H_2O between samples, drying completely with a paper towel.
7. Transfer homogenate to 1.5 mL Eppendorf tubes labeled H.
8. Centrifuge H at 200 × rcf for 5 min at 4°C to remove intact cells and debris.
9. Collect 50µL of supernatant in 1.5 mL Eppendorf tubes, add 50uL of 2X Sample Buffer (SB) with inhibitors and save as the **whole cell lysate (WCL).**
10. Boil for 5 min at 98°C then cool to room temperature and keep on ice.
11. Centrifuge H at 2,000 × rcf for 8 min at 4°C.
12. Transfer supernatant to a new 1.5 mL tube labeled MC and discard H.
13. Centrifuge MC at 2,000 × rcf for 8 min at 4°C.
14. Transfer supernatant to a new 1.5 mL tube labeled M and discard MC pellet.
15. Centrifuge M at 12,000 × rcf for 15 min at 4°C.
16. Collect supernatant without disturbing pellet in 1.5 mL tube labeled C1. Retain pellet as **mitochondrial fraction (M).**
17. Perform short spin of M pellet and aspirate remaining supernatant without disturbing the pellet. For liver samples, consider washing M tube with 500 µL of MIB, aspirate supernatant and perform the short spin. If pellet is disturbed during wash step, perform a second 12,000 × rcf spin for 15 min at 4°C.
18. Resuspend M pellet in 2X SB by pipetting up and down. For Skeletal muscle and Heart, add roughly $1/20^{th}$ of volume used for MIB in step 4. For Liver and Hippocampus, add roughly $1/10^{th}$ of volume.
19. Boil for 5 min at 98°C then cool to room temperature and keep on ice.
20. Centrifuge C1 at 20,000 × rcf for 15 min at 4°C.
21. Collect 100µL of supernatant in new 1.5 mL tube labeled **C2 (cytosolic fraction)** and add 100µL of 2X SB with inhibitors.
22. Boil for 5 min at 98°C then cool to room temperature and keep on ice.
23. Store all samples at -80°C once cooled to room temp if not using immediately. Consider aliquoting your samples to prevent freeze/thaw damage during repetitive uses.

Material Preparation:

- You will need 6 tubes per tissue for isolation of mitochondria. All should be labeled with Sample ID and tissue. Then, for each set of 6 label in order: **H, WCL, MC, M, C1, C2**. To identify total number of tubes needed, multiply N by number of tissues you will harvest per mouse, then multiply by 6.
- For homogenization, label 5 mL Falcon Round Bottom tubes with Sample ID and tissue.

Approximate MIB Volumes for various tissues:

Tissue	Approximate weight (mg)	Volume of MIB (uL)
Gastrocnemius (cut in half)	50	1000
Liver (roughly 1 square cm)	80	2000
Hippocampus (left & right)	25	500
Heart (1/3rd)	50	1000

Stock Reagent Preparation:

Mitochondria Isolation Buffer (MIB, 100 mL) – stock reagent

Reagent	Mol Weight (g/mol)	Product #	1 Liter (g)	[Final] mM
Mannitol	182.17	Sigma M4125	4.01	220 mM
Sucrose	342.30	Fisher BP220	2.40	70 mM
HEPES	238.30	Sigma H3375	0.476	20 mM
EDTA	292.23	Fisher S25687	0.0372	1 mM

1. Add approximately 80 mL of ddH_2O to a beaker.
2. Add the reagents in order listed above while stirring until fully dissolved. All reagents are in the chemical cabinet.
3. Adjust the pH to 7.4 using 1M KOH solution.
4. Bring final volume to 100 mL with ddH_2O.
5. Store at 4°C.

4.5X Sample Buffer, 44 mL

Reagent	Mol Weight (g/mol)	Product #	Location	Amount
0.5 M Tris-HCl			4°C	20 mL
Glycerol	92.09	Sigma G5516	Chem Cabinet	20 mL
SDS	288.38	Sigma L3771	Chem Cabinet	2 g
Bromophenol blue	669.96	403160050	Chem Cabinet	16 mg

1. Pipette 20 mL of 0.5 Tris-HCl into 100 mL beaker.
2. Transfer 20 mL of glycerol to beaker, rinse pipette completely by pipetting up and down.
3. Add SDS and Bromophenol blue.
4. Transfer to graduated cylinder and adjust volume to 44 mL with ddH_2O.
5. Store at 4°C.

4X Sample Buffer

Reagent	Mol Weight (g/mol)	Product #	Location	For 5 mL
4.5X SBSS			4°C	4.4 mL
1M DL-1,4-Dithiothreitol (DTT)	154.25		-20°C	0.4 mL
2-mercaptoethanol	78.13	ThermoFisher 125472500	Fume hood	0.2 mL

- Store at -20°C for up to 1 month.

Day of Reagent Preparation:

MIB with Inhibitors, 20 mL (~ 20 tissues)

Reagent	Product #	Location	For 20 mL
Stock MIB		4°C	19.6 mL
Protease Inhibitor Tablet	Roche 11836170001	4°C	2 Tablets
Phosphatase Inhibitor Cocktail 2	Sigma P5726	4°C	200 µL
Phosphatase Inhibitor Cocktail 3	Sigma P0044	4°C	200 µL

2X Sample Buffer + Phosphatase and Protease Inhibitors, 5 mL (~ 20 tissues)

Reagent	Product #	Location	For 5 mL
4X SB		-20°C	2.5 mL
Protease Inhibitor Tablet	Roche 11836170001	4°C	1 Tablet
Phosphatase Inhibitor Cocktail 2	Sigma P5726	4°C	100 µL
Phosphatase Inhibitor Cocktail 3	Sigma P0044	4°C	100 µL
ddH_2O			2.3 mL

- Vortex tablet and cocktails in ddH_2O until dissolved
- Add 2.5 mL 4X SB and invert to mix.
- Store at -20°C no longer than 1 week.