# Isolation of Mitochondria from Tissue via Differential Centrifugation.v2

This protocol is for isolation of mitochondria from tissues in mice based on a protocol from Ghosh et al (PLoS Comput Biol, 14, e1006640, 2018).

**Procedures**

1. Pre-cool the centrifuge to 4 ºC with "fast cool" feature.
2. Set up homogenization station.
    a. Place needed volumes of FRAC buffer on ice in 5 mL tubes.
    b. Chill a beaker of dd$H_2O$ to rinse homogenizer between samples and cool homogenizer on ice.
    c. Label 1.5 mL Tubes (Sample after homogenizing (1), Whole Cell (WC) fraction (1), cytosolic (cyto) fraction (3), mitochondria (mito) fraction (1))
3. Homogenize 1 mL per 100 mg tissue in 5 mL tube immersed in ice water bath using a polytron homogenizer. For 1 GA = 1.5 mL, but if only interested in mitochondrial fraction this volume can be increased to 2 mL. For ½ Heart = 1.5 mL FRAC buffer
4. Tranfer sample from 5 mL tube to 1.5 mL tube, vortex and take 100 uL and place in **WC** tube. Add 100 uL of 4X Sample buffer to 100 uL of WC (There is no need to have protease and phosphatase inhibitors as the FRAC buffer contains them) and boil for 4 min at 98°C for western blotting
5. Spin the remaining sample in 5 ml tube at 800 x g for 10 minutes at 4°C
6. Take resulting supernatant (containing mitochondria) and place in tube labeled **mito,** discard the pellet or save it for nuclear fraction if needed
7. Spin **mito** at 9000 x g for 10 minutes at 4°C
8. Remove resultant supernatant and place in **cyto** tube
9. **Keep mitochondrial pellet** in **mito** tube, and resuspend it in 200 uL of FRAC buffer
10. Spin mito samples at 11,000 x g for 10 min at 4°C
    *Optional: spin **cyto** fraction at 11,000 x g for 10 min at 4°C to further purify the cytosolic fraction from mitochondria contamination.
11. Add 100 uL of 4X Sample buffer to 100 uL of **cyto** fraction (There is no need to have protease and phosphatase inhibitors as the FRAC buffer contains them), then boil for 4 min at 98C for western blotting.
12. Resuspend **mito** pellet in 40 uL of 2X Sample buffer (150 µL for ½ heart as it contains more mitochondria). The volume of 2X Sample buffer is not rigid, depending on the size of the pellet.
13. Boil for 4 min at 98°C
14. Store all samples at -80°C

**Reagent Preparation:**

FRAC Buffer (pH 7.4) – storer ~1 month at 4°C

| Concentration | For 50 mL Stock |
|---|---|
| 21 mM HEPES | 260.27 mg |
| 250 mM Sucrose | 4.28 g |
| 0.1 mM EDTA | 1.46 mg |

1. Add HEPES to ~40 mL of dd$H_2O$ and let dissolve on stir plate
2. Add Sucrose and EDTA

3. Continuing stirring until completely dissolved
4. Adjust pH to 7.4 using 1 N HCl
5. Adjust final volume to 50 ML with $ddH_2O$
6. Store at 4°C for up to 1 month

4X Sample Buffer – store at -20°C

| Reagent | For 5 mL |
|---|---|
| 4.5X SBSS | 4.4 mL |
| 1M DL-1,4-Dithiothreitol (DTT) | 0.4 mL |
| 2-mercaptoethanol | 0.2 mL |

**Add inhibitor to buffers immediately before use**

FRAC Buffer (pH 7.4) + Phosphatase and Protease Inhibitors

| Reagent | For 5 mL Stock |
|---|---|
| FRAC Buffer | 4.8 mL |
| Protease Inhibitor Tablet | 1 Tablet |
| Phosphatase Inhibitor Cocktail 2 | 100 uL |
| Phosphatase Inhibitor Cocktail 3 | 100 uL |

2X Sample Buffer + Phosphatase and Protease Inhibitors

| Reagent | For 5 mL |
|---|---|
| 4X SB | 2.5 mL |
| Protease Inhibitor Tablet | 1 Tablet |
| Phosphatase Inhibitor Cocktail 2 | 100 uL |
| Phosphatase Inhibitor Cocktail 3 | 100 uL |
| $ddH_2O$ | 2.3 mL |

Note for making 2X SB:

- Dissolve (vortex) tablet and cocktails in small amount of $dH_2O$ (<1mL) first before adding anything else (no vortexing once SB is added due to SDS).
- Add 2.5 mL 4X SB into the tube and adjust final volume to 5 mL with $dH_2O$, invert to mix.

| Inhibitors | Product number |
|---|---|
| cOmplete mini, EDTA-free (protease inhibitor cocktail tablet) | Roche 11836170001 |
| Phosphatase Inhibitor Cocktail 2 | Sigma P5726 |
| Phosphatase Inhibitor Cocktail 3 | Sigma P0044 |