

Mitochondrial Isolation – Heart

Reference: Increased Uncoupling Proteins and Decreased Efficiency in Palmitate-perfused Hyperthyroid Rat Heart. Boehm, E.A., et al., Am. J. Physiol., 280: H977-H983, 2001.

BUFFERS:

Mitochondrial Isolation Medium (MIM):

Sucrose (300 mM)
Na-HEPES (10 mM)
EDTA (0.2 mM)
pH 7.2

MIM +BSA:

Sucrose (300 mM)
Na HEPES (10mM)
EDTA (0.2 mM)
BSA (1 mg/mL)
pH 7.4 (Note: pH differs from MIM)

Buffers can be prepared the day before and stored in the +4C refrigerator covered with parafilm or prepared in advance and frozen as follows:

For Frozen Aliquots:

Combine 82.15 g Sucrose, 2.08 g Na-HEPES and 8 mls of 20 mM EDTA, q.s. to 600 mls. Aliquot 30 mls into each of twenty 50 ml conical tubes and freeze at -20°C. Upon thawing each aliquot, the day before mitochondrial isolation either pH to 7.2 (with glacial acetic acid) and q.s. to 40 mls for MIM or add 1 mg/ml BSA, pH to 7.4 and q.s. to 40 mls for MIM+BSA.

Note that the protocol requires about 20 mls of MIM and 30 mls of MIM+BSA per sample processed.

For Buffer Preparation the Day Before Isolation (not using frozen aliquots):

Sucrose (300 mM) 41.08 g
Na-HEPES (10 mM) 1.04 g (Sigma H-3784, RT)
EDTA (0.2 mM) 0.030 g (Sigma E-5134, RT)

Initially QS to 300 mL and split into 150 mL aliquot for MIM and 150 mL aliquot for MIM+BSA.

MIM: pH to 7.2 (with glacial acetic acid), then QS to 200 mL, mix.

MIM+BSA : add 0.200 g BSA (Sigma A-8022, +4C dessicator), pH to 7.4, then QS to 200 mL, mix.

PREPARATION:

- Fill 2 ice buckets.
- Label two (non-sterile) 60mm dishes for each sample. Place about 10 mls MIM in one dish on ice per sample and place another labeled dry dish or lid on ice per sample.
- Prepare the following in 50 ml conical tubes and keep on ice:

Per sample:

Trypsin: 1.25 mg trypsin + 10 mls MIM (prepare in one tube for all samples)

Trypsin Inhibitor: 6.5 mg soybean trypsin inhibitor + 10 mls MIM+BSA

For two samples:

Trypsin: 0.0025 g trypsin (Sigma, T-9935, -20C dessicator) + 20 mls MIM

Trypsin Inhibitor: 0.0130 g soybean trypsin inhibitor (Sigma, T-9003, +4C
dessicator) + 20 mls MIM+BSA

ISOLATION:

- 1) Harvest tissue and place into dish with cold MIM on ice, rinsing off blood. Use 2-4 hearts per sample. (Use 4 hearts per sample if 3 week-old mice.)
- 2) In ice-cold MIM, trim and discard fat, atria, etc., saving biventricle.
- 3) Place tissue in 2nd (dry) dish on ice and mince finely (till hand hurts, approx 4-5 minutes, should look very homogeneous, should be able to pass easily through a 10ml pipet).
- 4) Add 10 mL of trypsin solution immediately after mincing the first sample, allowing the trypsin digestion to proceed on ice for 10 min exactly-use timer so while you are on the next sample you do not let the first sample over digest.
- 5) Pipet out of dish into labeled 50 ml conical tube.
- 6) Add 10 mL of trypsin inhibitor in MIM+BSA. Invert gently several times, allow tissue to settle, then discard supernatant.
- 7) Resuspend in 10 mls MIM+BSA on ice.
- 8) Homogenize (keeping dounce homogenizer on ice in a plastic beaker and avoiding bubbles!) with Eberbach device (speed is permanently set such that red handle aligns with large screw), about 5-7 passes (begin pass counting only after Teflon pestle fully reaches bottom of homogenizer) or until solution is uniform. Transfer back into labeled 15 ml conical.
- 9) Centrifuge suspension @ 600g for 10 min at 4°C (1650 rpm in IEC centrifuge).
- 10) Transfer resulting supernatant to a labeled Oakridge tube and centrifuge @ 8,000g for 15 min at 4°C (10,100 rpm in Beckman with JA-20 rotor).
- 11) Discard light-colored fluffy upper layer/supernatant.
- 12) Dislodge the remaining pellet with gentle pipet action and wash this dark, tightly packed mitochondrial pellet with 10 ml MIM+BSA on ice.
- 13) Re-spin @ 8,000g (10,100 rpm) for 15 min at 4°C, discard fluffy upper layer/ supernatant.
- 14) Gently direct 1 mL MIM from the pipet tip to the dry mitochondrial pellet, attempting not to disrupt the pellet, but rather to wash off the pellet any “light” or damaged

mitochondria, especially evident around the rim of the pellet. Aspirate off this wash containing the “light” mitochondria before the final suspension of the pellet in a very small amount of MIM as below. NOTE: Err on less volume since mitochondria should never be too dilute....they are not as stable.)

15) Re-suspend this final dense pellet in **MIM** (no BSA, to avoid confounding the protein assay). Depending on pellet size, this requires about 75 μL MIM per heart (150 μL MIM/ 2 hearts pooled; 225 μL MIM/ 3 hearts pooled.) Need to gently scrape pellet off tube wall with pipet tip. Transfer to 1.5 ml tube on ice and pipet gently to disrupt chunks and ensure even suspension prior to quantification.

16) Quantify mitochondrial protein employing the Pierce BCA protocol (see separate protocol). To quantify each sample, make three separate 1:50 dilutions of each mitochondrial isolate, removing 2 μL of the well-mixed mitochondrial isolate for addition to 98 μL of Diluent A in separate 1.5 mL tubes. Do this in triplicate to increase accuracy of this quantification since all respiration values will be normalized to mg of mitochondrial protein.

RESPIRATION (See separate protocol for more detail):

1) Set up computer and FOXY system as described in Respiration protocol.

2) Add 2 mls of Buffer C containing the desired substrate (see respiration protocol), then remove amount of liquid equal to calculated mitochondrial injection. Add 0.4 to 0.8 mg mitochondrial protein (typically, 0.5 mg) to the stirring 2 mL respiration chamber. Pipet mitochondrial isolate gently to ensure even suspension prior to removal of each mitochondrial aliquot for a respiration run. Final total chamber volume is 2 mls.

3) Respiration rates are faster with isolated mitochondria than permeabilized strips, and injections will be made rather quickly employing preloaded syringes. Oligomycin effects are seen almost immediately.

4) Units expressed as $\text{nmol O}_2/\text{min}/\text{mg}$ of mitochondrial protein. There is an Excel template in the lab which you should ask for in order to do your calculations.