Isolated Mitochondria Characterization

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We use this protocol and it's working

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ABSTRACT

This protocol is used to isolate mitochondria from cells. Isolated mitochondria can then be further analyzed, as described here, with a complex I enzyme activity assay, and/or blue native PAGE to quantify amounts of complex subunits.

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MATERIALS

Mitochondria Isolation

- EGTA (Sigma, #E4378)
- KOH (Sigma, CAS #1310-58-3)
- Sucrose (Sigma, #S0389)
- Tris-HCl (Thermo Fisher, #NC9348850)
- Mannitol (Sigma, #M4125)
- Sucrose (Sigma, #S0389)
- HEPES pH 7.4 (UCSF Media Core, CCFGL002)

Complex I Activity Assay

- 200 mM KCN (Sigma, # 60178; MW 65.12)
- 500 mM $MgCl_2$ (Sigma, #M8266-100G)
- 100 mg/mL BSA (Sigma, #A7030)
- 65 mM decylubiquinone (Sigma, #D7911; MW 322.44)
- 15 mM K_2NADH (Sigma, #N4505; MW 741.6)
- 1 mM Antimycin A (Sigma, #A8674; MW 548.63)
- 0.5 mM (Sigma, #R8875; MW 394.4)
- Spectrophometer for enzyme activity assay: Spectramax M4 platereader

Blue Native Page Gel

- Pierce™Coomassie Brilliant Blue Dyes (ThermoFisher, #20279)
- Nativepage 4x Sample Buffer (ThermoFisher, #BN2003)
- Nativepage 5% G250 (ThermoFisher, #BN2004)
- NativePAGE™Running Buffer (20X) (ThermoFisher, #BN2001)
- Nativepage 3-12% Gels (ThermoFisher, #BN1001)
- Total OXPHOS human western blot antibody cocktail (abcam, #ab110411)

Isolating Mitochondria

1 Make mitochondrial isolation buffers - starting buffer, mitochondrial resuspension buffer.

1.1 100 mM EGTA, pH 7.4:

- 3.8 g EGTA
- 70 mL water
- Adjusted pH KOH
- Bring to 100 mL with water

1.2 Starting Buffer (225 mM mannitol, 75 mM sucrose, 30 mM Tris-HCl, pH 7.4)

- 20.5 g mannitol
- 13 g sucrose
- 400 mL water
- 15 mL Tris-HCl (1 M, pH 7.4)
- Adjust pH to 7.4, and
- Bring to 500 mL with water

1.3 Mitochondrial Resuspension Buffer (250 mM mannitol, 5 mM HEPES pH 7.4, 0.5 mM EGTA)

- 4.56 g mannitol
- 80 mL water
- 1 mL 0.5M HEPES pH 7.4
- Allow to cool
- Adjust pH to 7.4
- Bring to 100 mL with water

2 Spin down 50 million cells.

3 Resuspend cells in 5 mL PBS to wash.

4 Spin down to create a cell pellet at 600 g for 5 minutes.

5 Aspirate out the supernatant.

6 Resuspend cells in 5 mL of PBS and spin down once more.

7 Aspirate off the PBS.

8 Resuspend the pellet in 1 mL of starting buffer and 100 mM EGTA (pH 7.4).

9 Split the cell solution into 2 chilled 2 mL tubes each containing half the cell solution.

10 Homogenize the cells using a tissue grinder for 200-250 strokes.

11 Transfer the homogenate to new chilled centrifuge tubes.

12 Centrifuge for 5 minutes at 600 g at 4˚C.

13 Resuspend the pellet containing mitochondria in starting buffer.

14 Centrifuge for 10 minutes at 7000 g at 4˚C.

15 Discard supernatant.

16 Resuspend the pellet in 1.6 mL starting buffer.

17 Centrifuge for 10 minutes at 10000 g at 4˚C.

18 Discard supernatant. Resuspend the pellet in 2 mL MRB buffer (mitochondria pellet).

19 Snap freeze samples by putting them on dry ice and spraying the ice down with 70% ethanol.

20 Store samples at -80˚C for further assays (i.e. enzyme activity assay, blue native PAGE).

Complex I Enzyme Activity Assay

21

> **Note**
>
> Only use ddH2O in this assay, as enzyme activity is affected by presence of detergent.

Make master mix with or without rotenone:

- 100 mM KPhos buffer pH 7.4 (make fresh monthly; store at 4°C)
- 200 mM KCN (**make fresh daily**; 0.0130 g/1 mL 1M KPhos buffer pH 7.0)
- 500 mM $MgCl_2$
- 100 mg/mL BSA
- 65 mM (21 mg/mL) decylubiquinone: Dissolve 10 mg of decylubiquinone in 0.476 mL ethanol

to make a 65 mM (21 mg/mL) stock for long-term storage at -20°C. On the day of the experiment, dilute 16 mL of 65 mM stock into 84 mL ethanol for a 100 mL working stock of ~10 mM

- 15 mM K_2NADH (**make fresh daily**; light sensitive; 0.0115 g/1 mL ddH_2O)
- 1 mM Antimycin A (store at -20°C; 5.486 mg/10 mL 200 proof EtOH)
- 0.5 mM Rotenone (store at -20°C; light sensitive; 5.916 mg/30 mL 200 proof EtOH)

22 Dispense 100 µL of master mix into designated wells of a 96 well plate.

23 Set spectrophometer (Spectramax M4 platereader) to read 340 nm wavelength for 5 min at constant 37°C.

24 QUICKLY dispense 10 µg of crude mitochondria preps into each master mix well (minimize time here!).

25 Record reading on spectrophotometer for 45 min.

Blue Native Page

26 Prepare 20 µl sample buffer cocktail for every 50 µg crude mitochondria prep:

- 5 µl of NativePAGE sample buffer (4×), 8 µl of 5% digitonin, and 7 µl of water for a 8g/g digitonin to protein ratio.

27 For dark blue cathode buffer, dissolve 0.044 g Coomassie Brilliant Blue G-250 in 220 ml of Native PAGE anode buffer, mix well.

28 For light blue cathode buffer, add 20 ml of dark blue cathode buffer into 180ml of native PAGE anode buffer, mix well. The buffer can only be used once.

29 Solubilize mitochondria by adding 20 µl sample buffer cocktail to 50 µg crude mitochondria prep and gently mix with a P20 pipette. Make sure to avoid formation of bubbles.

30 Incubate solubilized mitochondria on ice for 20 min.

31 Centrifuge at 20,000 × *g* for 10 min at 4°C and then collect 15 µl of the supernatant into new tubes.

32 Add 2 µl of Coomassie G-250 sample additive to the above supernatant. The G-250 volume should be such that the final G-250 concentration is 1/4th the detergent concentration. This step should be done just before loading the samples into the gel.

33 Set up the electrophoresis system (XCell SureLock Mini-Cell):
- Remove the white tape on the bottom of a NativePAGE 3-12% gradient gel and place it in the apparatus.

34 Wash the wells of the gel with 1 ml of dark blue cathode buffer. Put 20 ml of dark blue cathode buffer in the inner chamber and check for leakage.

35 Load 15 µl of sample into the gel by using Prot/Elec Tips. Fill the inner chamber with about 180 ml of dark blue cathode buffer, and then fill the outside chamber with about 600 ml of running buffer.

36 Carefully add the dark blue cathode buffer without disturbing the samples in wells.

37 Turn on the power supply and run the gel at 150 V for 30 min. Remove the dark blue buffer with a 10-ml size pipette or suction tube and fill the inner chamber with 200 ml of light blue buffer. Run the gel at 250 V for 60 min. If better separation of the bands is desired then the gel can be run for maximum of 150 min at 250 V. A longer run time than 150 min has minimal impact on the separation and resolution of bands.

38 To transfer the BN-PAGE gel, first take the gel out of the cassette and wash it with water.

39 Incubate the gel in transfer buffer for 15 min with gentle shaking, before transferring gel to PVDF membrane using an iBlot Gel Transfer Device.

40 Block and stain PVDF membrane with total OXPHOS human western blot antibody cocktail at 1:500 concentration.

41 Visualize blots with an Odyssey infrared imaging system.

42 Analyze blots with Image Studio Lite Software from Li-cor Biosciences.