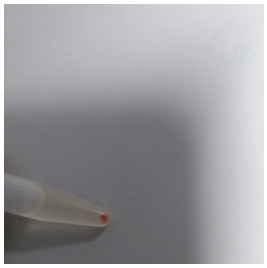
Mar 13, 2025

Mitochondrial Sub-fractionation Using Differential Centrifugation - Cells



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Protocol status: Working
We use this protocol and it's working

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Disclaimer

NA

Abstract

Crude fractionation of cells to yield crude mitochondria and subfractions using differential centrifugation

Guidelines

GM cells should be treated according to local safety guidelines

Materials

Eppendorf tubes
Dounce tissue grinder (loose and tight fitting)
Tris
EDTA
Sucrose
Centrifuge capable of 10,000g
Ultracentrifuge (optional)

Protocol materials

1X PBS (Phosphate-buffered saline)

Safety warnings

No warnings

Ethics statement

NA

Before start

Prepare and cool the buffers
Prepare ice

Process collected cells and prepare crude mitochondria

2h 5m

1 Start with a cell pellet (for crude mitochondrial preparation start with a minimum of a confluent 10cm dish, for sub-fractionation start with minimum of 3-6 confluent T75cm flasks). The cell pellet is always kept cold On ice

2 Wash the cell pellet once with 1X PBS (Phosphate-buffered saline) and 1200 rpm, 21°C, 00:05:00 to generate a washed cell pellet. On ice

5m

3 Dislodge the cell pellet with approximately 1000 µL of TES buffer (70 mM Tris base, 1mM EDTA, 0.25 M sucrose, pH 7.4) and transfer to a loose fit hand held homogenizer (such as Eppi-pestles +1.5mL tube). With strong force by hand, turn the pestles ten times. Transfer the homogenate to a tight fitting glass homogenizer (such as dounce tissue grinder Sigma-Aldrich P1110-1EA) and with strong force by hand dounce the homogenate by turning the glass pestle as you move up and down in the glass mortar tube 10 times. On ice

Equipment

Dounce Tissue Grinder Set with Two Glass Pestles	NAME
Wheaton	BRAND
cat# 357538	SKU
https://us.vwr.com/store/product/4832641/dounce-tissue-grinders-wheaton	LINK

Equipment

Eppi-pestles	NAME
Pestle for 1.5mL tube	TYPE
Schuett biotec	BRAND
3200512	SKU
https://www.schuett-biotec.de/	LINK

4 Transfer the homogenate to a 2mL round bottomed "Eppendorf" tube (Eppi). Optional: aliquot a small amount of the homogenate in a new tube labelled "TE" total extract or homogenate. On ice

5 1000 x g, 4°C, 00:10:00 10m

6 Carefully remove the supernatant and transfer to a fresh 1.5mL Eppi and label "M" On ice . Add 500µL TES buffer to the "nuc" pellet and homogenize again (see above). 1000 x g, 4°C, 00:10:00 . Transfer the supernatant to the "M" tube on ice. Label the nuc pellet tube "N" and store if needed. On ice 10m

7 Tube "M" > 9800 x g, 4°C, 00:15:00 15m

8 Transfer the supernatant to a new Eppi labelled "C" for cytosolic fraction. On ice

9 Look carefully at the "M" pellet which is crude mitochondria. The pellet should be red/brown/yellow in colour and flaky/hydrophobic (see image in tab "description" of this protocol). If the mitochondrial pellet is surrounded by contamination (white fluffy pellet),

the contaminating white fluffy pellet can be carefully removed with a pasteur pippette pump or a pipette.

10 Optional: wash the mitochondrial pellet in TES buffer and centrifuge again 10m
9800 x g, 4°C, 00:10:00

11 Resuspend the mitochondrial pellet in TMG buffer (10 mM Tris, 5 mM MgCl2, 20 % (v/v) glycerol) and perform protein estimation. Working range: minimum 0.7mg/mL mitochondrial protein concentration for most applications and assays. On ice

12 Optional: aliquot a small amount of the crude mitochondrial extract. On ice

13 Optional: to store the crude mitochondrial fraction for further use; 15m
9800 x g, 4°C, 00:15:00 and store as a dry pellet at -80 °C

Mitochondrial sub-fractions 10m

14 Optional: To immunoprecipitate from crude mitochondria, first resuspend the mitochondrial pellet in TES buffer and sonicate for 1 min at 5s intervals (14uA amplitude)
On ice

15 Incubate the crude mitochondrial fraction in ice cold digitonin (0.2 mg/mg mitochondrial protein) rolling or turning at 4 °C for 15 minutes.

16 9800 x g, 4°C, 00:10:00 10m

17 Pellet = mitoplast (IMM and matrix). Supernatant = OMM/IMS.

18 Optional: store supernatant or pellet resuspended in TES at -80 °C

Further fractionation 1h

19 Disrupt mitoplast by sonicating for 1 min at 5s intervals (14uA amplitude) On ice . Do the
same for the OMM/IMS fraction On ice .

20 Centrifuge all disrupted fractions: mitoplast, OMM/IMS and if needed cytosolic fraction at high speed. 100000 x g, 4°C, 01:00:00 1h

21 Mitoplast: Supernatant = matrix, pellet = IMM
OMM/IMS: Supernatant = IMS, pellet = OMM
Cytosol: Supernatant=purified cytosol, pellet = membrane contaminants.

22 Resuspend pellets in 100-200 µL TES and store all fractions at -80 C or use straight away.

Acknowledgements

Modified after a protocol developed for tissue by Prof. Ellen Billett (retired). Nottingham Trent University, UK