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Assessing enrichment of proteins in the mitochondrial fraction in HEK cells

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ABSTRACT

This is a method for measuring protein enrichment on mitochondria in various conditions. In the resulting Western blot, one can assess the level of contamination of other organelles in the enrichment prep.

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GUIDELINES

Up to 6 samples can be processed at one time.

MATERIALS TEXT

ThermoScientific Mitochondrial Enrichment Kit for Cultured Mammalian Cells
Pierce BCA Protein Assay Kit
LICOR Western Blotting Reagents
LICOR Odyssey Blot Scanner
ImageStudio Software

BEFORE STARTING

Add protease and phosphatase inhibitors such as Pepstatin A, TAME, Leupeptin, DTT, and PMSF) to Reagents A and C (800 uL Reagent A and 1300 uL Reagent C needed per sample)
Chill all reagents and tools on ice before starting.

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Cell Lysis and Mitochondrial Enrichment

1 Place dish of cells on ice and gently aspirate media.

2 Add 800 uL cold PBS and scrape cells into a 2 mL tube

2.1 If using multiple plates per condition, combine all cells into 800 uL PBS.

3 Save 50 uL of cell suspension for whole cell lysis (Input).

4 Centrifuge both tubes at 850g for 2 min at 4 degrees C.

5 Aspirate supernatants from both tubes.

6 For Input sample, snap freeze in liquid nitrogen and store at -20 degrees C for later lysis.

7 For bulk of cells, add 800 uL Reagent A with protease and phosphatase inhibitors.

8 Vortex 5 sec and incubate on ice for 2 min

9 Add 10 uL Reagent B

10 Incubate on ice for 5 min, vortexing every minute.

11 Add 800 uL Reagent C with protease and phosphatase inhibitors

12 Invert by hand to mix

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13 Centrifuge at 700 g for 10 min at 4 degrees C.

14 Transfer supernatant to a new tube

15 Centrifuge at 3,000 g for 15 min at 4 degrees C

16 Transfer supernatant (cytosolic fraction) to a new tube and save on ice.

17 Add 500 uL Reagent C with protease and phosphatase inhibitors to the pellet

18 Centrifuge 12,000 g for 5 min at 4 degrees C

19 Save supernatant as Wash.

20 Suspend final pellet in 80 uL RIPA buffer with protease and phosphatase inhibitors by vigorous pipetting and vortexing.

21 Save sample on ice or at -20 degrees C. Minimize freeze/thaw cycles.

Prepare Input lysis

22 Thaw snap-frozen whole cell sample on ice for 5 min

23 Add 100 uL RIPA buffer with protease and phosphatase inhibitors

24 Incubate on ice 20 min

25 Centrifuge 20 min at 17,000 g at -20 degrees C

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26 Save supernatant as Input

Determine protein concentration of enrichment, cytosol, and input samples

27 Perform a BCA assay with Pierce kit or similar using 10 uL sample per well in duplicate

Gel Electrophoresis and transfer

28 Add denaturing buffer to samples

29 Load 30 ug of each sample onto acrylamide gels along with a molecular weight standard for each set

29.1 30 ug into 10% gel and 30 ug into 14% gel for each sample

30 Run samples and transfer resulting protein array to PVDF membrane

Membrane blotting

31 Stain total protein and image with preferred method

32 Cut membranes to produce sections for the respective proteins:
(10% set)
90 kDa - 260 kDa (LAMP1, TBK1)
40 kDa - 70 kDa (Parkin)
25 kDa - 38 kDa (GAPDH)

(14% set)
8 kDa - 25 kDa (TOMM20)

33 Clear total protein stain

34 Block membranes

35 Incubate membranes with primary antibodies for the respective proteins overnight at 4 degrees.

90 kDa - 260 kDa : LAMP1, TBK1

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40 kDa - 70 kDa : Parkin
25 kDa - 38 kDa : GAPDH

(14% set)
8 kDa - 25 kDa : TOMM20

36 Wash membranes 4x 5 min with TBS and 0.2% TWEEN

37 Incubate membranes with LICOR infrared spectrum secondary antibodies to the respective species

38 Wash membranes 4x 5 min with TBS and 0.2% TWEEN

39 Image membranes with Odyssey scanner

Quantification

40 Use ImageStudio sofware to outline bands of interest for each sample with the Box function

41 Assess GAPDH bands in Enrichment fraction to determine amount of cytosolic contamination. Assess LAMP1 bands to determine amount of lysosomal contamination.

42 Transfer band intensities after background subtraction to an Excel document

43 Divide band intenities from the Enrichment fraction by their corresponding intensities in the Input section to determine the enrichment efficacy.

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