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Crude Membrane Fractionation of Cultured Cells

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We use this protocol and it's working

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Abstract

We present here a protocol for fractionating crude cellular extracts to prepare membrane and cytosol-enriched fractions and a nuclei-containing insoluble fraction from cultured cells. We deploy this protocol for determining the membrane versus cytosolic distribution of components from LRRK1 and LRRK2 signaling pathways.

Note

We recommend analysing the products of this fractionation scheme by quantitative immunoblotting (as described indx.doi.org/10.17504/protocols.io.6qpvr68e3vmk/v1).

Note

This protocol was adapted from https://doi.org/10.15252/embj.201798099

Attachments

DOCX

466-976.docx
781KB

Materials

MATERIALS

Reagents:

Buffer A:

[M] 10 millimolar (mM) HEPES pH 7.4 and cOmplete^{TM} EDTA-free Protease Inhibitor Cocktail

Note

Added fresh before use, cOmplete™, Mini, EDTA-free (Protease Inhibitor) **Roche Catalog** ##11836170001)

Buffer B:

	A	B
	HEPES pH 7.4	250 mM
	Sodium Chloride	750 mM
	Magnesium Chloride	25 mM
	DTT	2.5 mM
	GDP	500 nM
	Sodium Fluoride	250 mM
	Sodium Pyrophosphate	25 mM
	Microcystin-LR (Enzo Life Sciences, ALX-350-012)	5 µg/ml
	cOmpleteTMEDTA-free Protease Inhibitor Cocktail (added fresh before use, Roche, 11836170001)	

Note

This buffer is prepared at a 5X stock to achieve a final concentration of 1X in the resuspension buffer (4 X Buffer **A** + 1 X Buffer **B)**.

Buffer C:

	A	B
	HEPES pH 7.4	50 mM
	Sodium Chloride	150 mM
	Magnesium Chloride	5 mM
	DTT	0.5 mM
	GDP	100 nM
	Sodium Fluoride	50 mM
	Sodium Pyrophosphate	5 mM
	Microcystin-LR (Enzo Life Sciences, ALX-350-012)	1 µg/ml
	Triton X-100	1% (v/v)
	cOmpleteTMEDTA-free Protease Inhibitor Cocktail (added fresh before use, Roche, 11836170001)	

- Gibco™ PBS pH 7.4 **Thermo Fisher Scientific Catalog #**10728775
- Pierce™ Coomassie Plus (Bradford) Assay Kit **Thermo Fisher Catalog #**23236 or equivalent).
- 4X Loading buffer:

  NUPAGE LDS sample buffer (4x) **Thermo Fisher Scientific Catalog #**NP0007 or 4X SDS loading buffer:

	A	B
	Tris-HCl, pH6.8	250mM
	SDS	8% (w/v)
	Glycerol	40% (v/v)
	Bromophenol blue	0.02% (w/v)

Reagents and antibodies:

Rubber tipped scraper **Merck MilliporeSigma (Sigma-Aldrich) Catalog #**CLS3008

Anti-Rab7 antibody Mouse monoclonal **Merck MilliporeSigma (Sigma-Aldrich) Catalog #**R8779

Recombinant Anti-RAB7 (phospho S72) antibody [MJF-R38-1] (ab302494) **Abcam Catalog #**ab302494

Recombinant Anti-PKC alpha antibody [Y124] (ab32376) **Abcam Catalog #**ab32376

Recombinant Anti-Sodium Potassium ATPase antibody [EP1845Y] - Plasma Membrane Loading Control **Abcam Catalog #**ab76020

Anti-Rab7 antibody Mouse monoclonal **Merck MilliporeSigma (Sigma-Aldrich) Catalog #**R8779

α-Tubulin Antibody **Cell Signaling Technology Catalog #**2144

Phospho-p44/42 MAPK (Erk1/2) (Thr202/Tyr204) Antibody **Cell Signaling Technology Catalog #**9101

Equipment:

Equipment	
Corning® cell lifter	NAME
Corning® cell lifter	BRAND
CLS3008	SKU
https://www.sigmaaldrich.com/IN/en/product/sigma/cls3008	LINK
Blade L 19 mm, handle L 180 mm, sterile, case of 100	SPECIFICATIONS

or equivalent

Equipment

Eppendorf™ 5810R Centrifuge	NAME
Centrifuge	TYPE
Eppendorf	BRAND
02-262-8187	SKU
https://www.fishersci.com/shop/products/eppendorf-5810r-centrifuge-rotor-packages-16/022628187	LINK

or equivalent

Equipment

Eppendorf® microcentrifuge	NAME
Centrifuge	TYPE
Eppendorf®	BRAND
5417	SKU
https://www.sigmaaldrich.com/IN/en/product/sigma/z366013?gclid=CjwKCAjwtIaVBhBkEiwAsr7-cwVnwbWrBxOUW73qAEiKOBI00UeWVtRsRmiqBWQxzgNG-e_2iC6UNRoCZKAQAvD_BwE	LINK

or equivalent

Equipment

Luer Slip 1ml IV Syringes (Medicina IVS01)

NAME

Medicina Luer Slip IV Syringes can be used with any standard, filtered or safety needles.

TYPE

Medicina

BRAND

IVS01

SKU

https://medicina.co.uk/luer-slip-iv-syringes/

LINK

or equivalent

Equipment

25G Luer Needle (TerumoTM NN-2525R)

NAME

Terumo Hypodermic Needles

TYPE

Terumo

BRAND

NN-2525R

SKU

https://www.terumotmp.com/products/hypodermics/terumo-hypodermic-needles.html

LINK

or equivalent

Equipment

Thick-walled Polycarbonate Tubes (Beckman Coulter 343775)	NAME
Thick-walled Polycarbonate Tubes	TYPE
Beckman Coulter	BRAND
343775	SKU
https://www.mybeckman.in/supplies/tubes-and-bottles/open-top-tubes/343775	LINK

or equivalent

- Ultracentrifuge (Beckman Coulter Optima TLX, or equivalent)
- Ultracentrifuge rotor (Beckman Coulter TLS.55 or equivalent)
- Plate reader for Protein quantification (BioTek Epoch, or equivalent)

Troubleshooting

Crude Membrane Fractionation

1

Note

The optimal quantity of cultured cells to use to achieve an ideal yield will vary dependent on cell type. As a guideline, we use 1 x 15cm dish of HEK293 cells per replicate seeded at 1.8 x 10^7 cells per dish.

Pour off media from the culture dish and aspirate completely by holding plate on edge. Wash cells twice with 5 mL of ice-cold PBS.

2 Immediately transfer the dishes to ice--this is best accomplished using wet paper towel-covered steel blocks resting On ice .

3 Add 5 mL of ice-cold PBS and scrape the cells from the dish using a cell lifter (Sigma-Aldrich CLS3008, rubber tipped scraper, or equivalent) to ensure good yield; collect in a 15 ml tube.

4 Pellet intact cells by centrifugation at 100 x g for 00:05:00 at 4 °C and aspirate supernatant. 5m

5 Resuspend cells in 400 µL of Buffer **A** by gentle pipetting.

5.1 Transfer to an 1.5ml Eppendorf tube and incubate On ice for 00:15:00 . 15m

Note

Note that this is a hypotonic solution and will swell the cells; 00:05:00 . is likely sufficient at this stage.

6 Add 100 µL of cold Buffer **B** to the cell suspension.

7 Using a 25-gauge needle attached to a 1 ml syringe, break the cells by passing the cell suspension through the needle 25 times.

Note

Breakage can be monitored by transferring a few microliters of the homogenate to a glass slide, covering with a coverslip and visualizing using a low power light microscope used to visualize cultured cells; as few as 6-10 passages may be sufficient. Broken cells will lose their reflective character and small particles of cell components will be readily detected.

8 Centrifuge the cell suspension at 1000 x g for 00:05:00 at 4 °C and collect the supernatant in a new 1.5ml Eppendorf tube. 5m

Note

The pellet here will contain the nuclei and other cell debris. This can be analysed by lysing in 500 µL Buffer **C**. The supernatant represents the post-nuclear supernatant.

9 Load the post-nuclear supernatant into thick-walled polycarbonate tubes, appropriate for ultracentrifugation in a table top ultracentrifuge. Ultracentrifuge at 150000 x g for 00:20:00 at 4 °C . 20m

Note

The membrane pellet will form at the bottom of the tube.

10 Transfer the cytosolic fraction (supernatant) to a fresh Eppendorf tube On ice .

11 Wash the membrane fraction pellet will 500 µL PBS thrice to remove any potential cytosolic contaminants.

Note

This may not be necessary if aspiration is complete.

12 Resuspend membrane pellet using 500 µL of Buffer **C** using a pipet and incubate On ice for 00:05:00 00:20:00 to allow detergent solubilization of 25m

membrane proteins.

13 Centrifuge membrane protein solution at 1000 x g for 00:05:00 at 4 °C to separate solubilized membrane proteins (supernatant) from insoluble membrane proteins (pellet). 5m

14 Determine the protein concentration of cell lysates by Bradford assay according to the manufacturer's instructions, performing measurements in triplicate.

Note

Ensure the concentration of the samples is in the linear range for the Bradford assay. If it isn't, prepare appropriate dilutions in water of each lysate. Generally, protein concentrations of near confluent cells lysed as described above should result in protein concentrations of at least [M] 2 µg/µL .

15 4×SDS–PAGE sample buffer is added to samples containing 5 µg of membrane protein or an equivalent volume of cytosolic protein, and heated at 37 °C for 00:10:00 . 10m

Analysis of fractionation products by quantitative immunoblotting analysis

1d 16h 30m

16 The reaction products can be analysed by quantitative immunoblotting analysis (as described in dx.doi.org/10.17504/protocols.io.6qpvr68e3vmk/v1).

	A	B	C	D	E
	Antibody Target	**Company**	**Cat. number**	**Host species**	**Dilution**
	pS72 Rab7A	Abcam Inc.	ab302494	Rabbit	1:1000
	Rab7A (Total)	Sigma	R8779	Mouse	1:2000
	LRRK1 (total) (C-terminus)	MRC-PPU Reagents and Services, University of Dundee	S405C	Sheep	1 µg/ml
	Tubulin	Cell Signaling Technologies	2144	Mouse	1:5,000

	A	B	C	D	E
	pT202/Y204 ERK1/2	Cell Signaling Technologies	9101	Rabbit	1:1000
	PKCα	Abcam Inc.	ab32376	Mouse	1:1000
	Na-K ATPase	Abcam Inc.	ab76020	Rabbit	1:10,000

17 1d

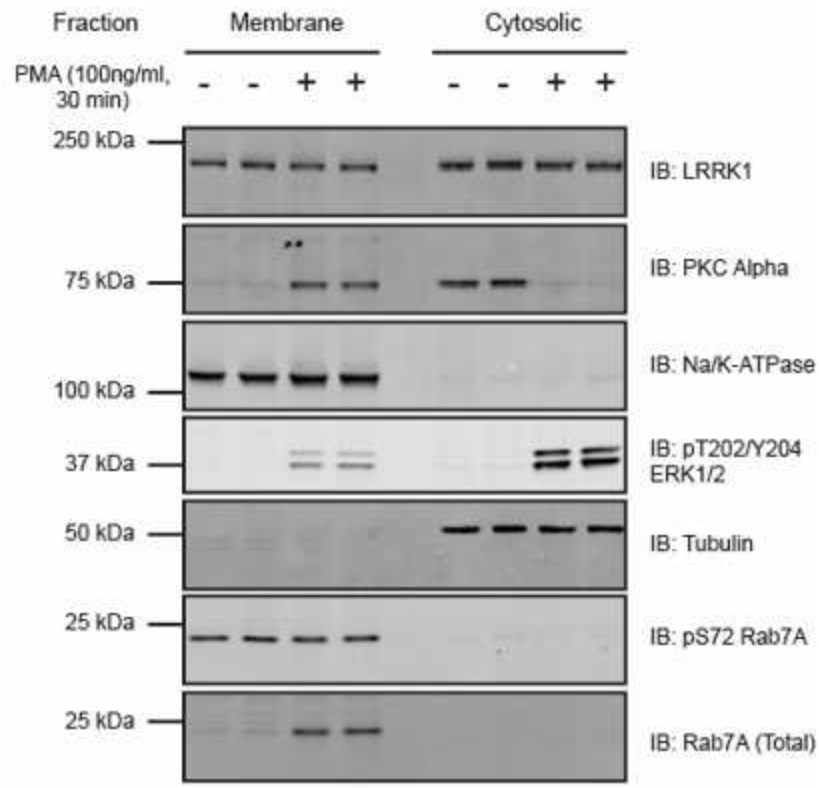

Figure 1: Crude membrane fractionation of HEK293 Flp-in T-REx/GFP-LRRK1 WT cells following phorbol ester stimulation.

HEK293 Flp-in T-REx/GFP-LRRK1 WT cells were induced to express GFP-LRRK1 wild type by treatment with [M] 1 mg/mL doxycycline for 24:00:00 .

18 Serum starve the cells for 16:00:00 and then treated ± Phorbol myristic acid (PMA) ([M] 100 ng/ml) for 00:30:00 . 16h 30m

19 Following this, Perform the fractionation as described here and samples were subjected to immunoblot analysis with the indicated antibodies; the membranes were visualized using the Odyssey CLx scan Western Blot imaging system.

Note

Adapted from https://doi.org/10.1101/2022.06.09.495448.