Aug 12, 2022

Crude Membrane Fractionation of Cultured Cells

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dx.doi.org/10.17504/protocols.io.yxmvmnb99g3p/v1

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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.

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

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

ATTACHMENTS

466-976.docx

DOI

dx.doi.org/10.17504/protocols.io.yxmvmnb99g3p/v1

PROTOCOL CITATION

Asad Malik, Dario R Alessi, Suzanne R Pfeffer 2022. Crude Membrane Fractionation of Cultured Cells. **protocols.io** https://protocols.io/view/crude-membrane-fractionation-of-cultured-cells-cbfgsjjw

KEYWORDS

Cultured Cells, Crude Membrane Fractionation, Immunoblotting analysis

CREATED

Jun 16, 2022

LAST MODIFIED

Aug 12, 2022

OWNERSHIP HISTORY

Jun 16, 2022 maria.s

Jun 20, 2022 Dario R Alessi

PROTOCOL INTEGER ID

64712

MATERIALS TEXT

MATERIALS

Reagents:

Buffer A:

10 millimolar (mM) HEPES **7.4** and cOmplete™ EDTA-free Protease Inhibitor Cocktail

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)	

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:

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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 **Sigma-aldrich Catalog #CLS3008**

☒ Anti-Rab7 antibody Mouse monoclonal **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 **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:

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Corning® cell lifter

Corning® cell lifter **CLS3008**

Blade L 19 mm, handle L 180 mm, sterile, case of 100

or equivalent

Eppendorf™ 5810R Centrifuge

Centrifuge

Eppendorf **02-262-8187**

or equivalent

Eppendorf® microcentrifuge

Centrifuge

Eppendorf® **5417**

or equivalent

Luer Slip 1ml IV Syringes (Medicina IVS01)

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

Medicina **IVS01**

or equivalent

25G Luer Needle (TerumoTM NN-2525R)

Terumo Hypodermic Needles

Terumo **NN-2525R**

or equivalent

Thick-walled Polycarbonate Tubes (Beckman Coulter 343775)

Thick-walled Polycarbonate Tubes

Beckman Coulter **343775**

or equivalent

- Ultracentrifuge (Beckman Coulter Optima TLX, or equivalent)
- Ultracentrifuge rotor (Beckman Coulter TLS.55 or equivalent)

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- Plate reader for Protein quantification (BioTek Epoch, or equivalent)

Crude Membrane Fractionation

1

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 5m

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

5

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

5.1 15m

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

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.

Breakage can be monitored by transferring a few microliters of the homogenate to a glass slide, covering with a

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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 5m

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.

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 20m

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** .

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.

This may not be necessary if aspiration is complete.

12 25m

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 membrane proteins.

13 5m

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).

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

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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 **2 µg/µL** .

15 10m

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** .

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
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

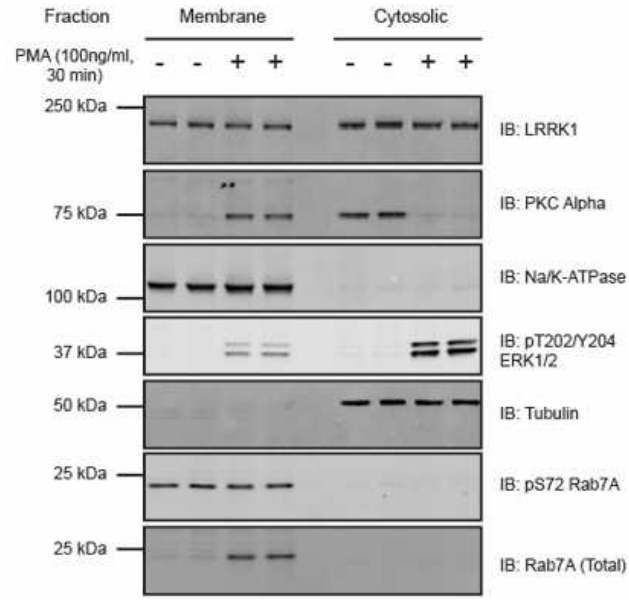

Figure 1: Crude membrane fractionation of HEK293 Flp-in T-REx/GFP-LRRK1 WT cells following phorbol ester stimulation.

HEK293 Flp-in T-REx/GFP-LRRK1 WT cells were induced to express GFP-LRRK1 wild type by treatment with

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[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 16h 30m **00:30:00** .

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.

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

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