**Crude Membrane Fractionation of Cultured Cells**

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**Summary**

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 signalling pathways.

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

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

**1) MATERIALS**

**1.1) Reagents:**

1.1.1) Buffer **A**: 10 mM HEPES pH 7.4 and cOmpleteTM EDTA-free Protease Inhibitor Cocktail (added fresh before use, Roche, 11836170001)

1.1.2) Buffer **B**: 250 mM HEPES pH 7.4, 750 mM Sodium Chloride, 25 mM Magnesium Chloride, 2.5 mM DTT, 500 nM GDP, 250 mM Sodium Fluoride, 25 mM Sodium Pyrophosphate, 5 μg/ml Microcystin-LR (Enzo Life Sciences, ALX-350-012)1, cOmpleteTM EDTA-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)**

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

1.1.3) Phosphate buffered saline (PBS), pH 7.4 (ThermoFisher Scientific #10728775)

1.1.4) Bradford assay kit (Pierce™ Coomassie Plus (Bradford) Assay Kit, ThermoFisher Scientific 23236, or equivalent).

1.1.5) 4X Loading buffer: Invitrogen™ NuPAGE™ LDS Sample Buffer (#NP0007), or 4X SDS loading buffer: 250mM Tris-HCl, pH6.8, 8% (w/v) SDS, 40% (v/v) glycerol, 0.02% (w/v) bromophenol blue.

**1.2) Equipment:**

1.2.1) Cell lifter (Sigma-Aldrich CLS3008, or equivalent)

1.2.2) Refrigerated bench-top centrifuge (Eppendorf centrifuge 5810R, or equivalent)

1.2.3) Refrigerated bench-top centrifuge (Eppendorf microcentrifuge 5417R, or equivalent)

1.2.4) Luer Slip 1ml IV Syringes (Medicina IVS01, or equivalent)

1.2.5) 25G Luer Needle (TerumoTM AN-2525R, or equivalent)

1.2.6) Thick-walled Polycarbonate Tubes (Beckman Coulter 343775, or equivalent)

1.2.7) Ultracentrifuge (Beckman Coulter Optima TLX, or equivalent)

1.2.8) Ultracentrifuge rotor (Beckman Coulter TLS.55 or equivalent)

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

**2) METHOD:**

**2.1) Crude Membrane Fractionation**

*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 107 cells per dish.

2.1.1) 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.

2.1.2) Immediately transfer the dishes to ice--this is best accomplished using wet paper towel-covered steel blocks resting on ice

2.1.3) 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

2.1.4) Pellet intact cells by centrifugation at 100 x g for 5 min at 4oC and aspirate supernatant

2.1.5) Resuspend cells in 400 µl of Buffer **A** by gentle pipetting; transfer to an 1.5ml Eppendorf tube and incubate on ice for 15 min. [Note that this is a hypotonic solution and will swell the cells; 5 min. is likely sufficient at this stage].

2.1.6) Add 100 µl of cold Buffer **B** to the cell suspension

2.1.7) 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.

2.1.8) Centrifuge the cell suspension at 1000g for 5 min at 4oC and collect the supernatant in a new 1.5ml Eppendorf tube

*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.

2.1.9) Load the post-nuclear supernatant into thick-walled polycarbonate tubes, appropriate for ultracentrifugation in a table top ultracentrifuge. Ultracentrifuge at 150,000g for 20 min at 4oC.

Note: The membrane pellet will form at the bottom of the tube

2.1.10) Transfer the cytosolic fraction (supernatant) to a fresh Eppendorf tube on ice.

2.1.11) 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.]

2.1.12) Resuspend membrane pellet using 500 µl of Buffer **C** using a pipet and incubate on ice for 5-20 min to allow detergent solubilization of membrane proteins.

2.1.13) Centrifuge membrane protein solution at 1000g for 5 min at 4 oC to separate solubilized membrane proteins (supernatant) from insoluble membrane proteins (pellet).

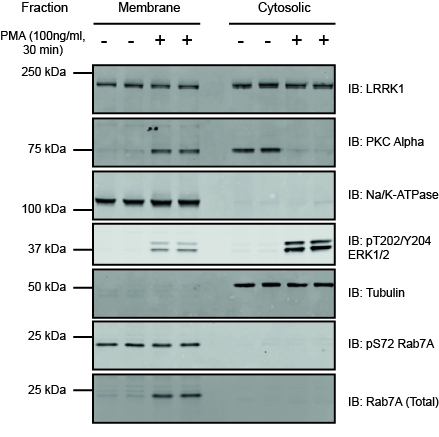
2.1.14) Determine the protein concentration of cell lysates by Bradford assay according to the manufacturer’s instructions, performing measurements in triplicate.

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 2µg/µl.

2.1.15) 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 10 min

**2.2) Analysis of fractionation products by quantitative immunoblotting analysis:** The reaction products can be analysed by quantitative immunoblotting analysis (as described in dx.doi.org/10.17504/protocols.io.6qpvr68e3vmk/v1).

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| **­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 |

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**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 1mg/ml doxycycline for 24 h. Cells were then serum starved for 16 h and then treated ± Phorbol myristic acid (PMA) (100 ng/ml) for 30 min. Following this, fractionation was performed 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.