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### OPEN ACCESS

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

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**Keywords:** Lysate, Immunoblot, Western, Phospho, LRRK2

# © Cell lysis and immunoblotting for protein and phosphoprotein quantification

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**ABSTRACT** 

Here, we describe the procedure by which human iPSC-derived neurons or mouse embryonic fibroblasts (MEFs) were lysed and probed for levels of proteins of interest using Western blot.

**ATTACHMENTS** 

552-1148.pdf

**MATERIALS** 

#### Reagents

#### RIPA buffer

А	В
Tris-HCl	50 mM
NaCl	150 mM
Triton X-100	0.1%
Deoxycholate	0.5%
SDS	0.1%

- HALT phosphatase and protease inhibitor cocktail (100x) Thermo Fisher Scientific Catalog #78442
- Microcystin-LR Microcystis aeruginosa CAS 101043-37-2 Calbiochem Merco MilliporeSigma (Sigma-Aldrich) Catalog #475815m
- Pierce BCA Protein Assay Kit Thermo Fisher Scientific Catalog #23225

#### 4x Protein Loading Buffer

	A	В
	Tris-HCl, pH 6.8	125 mM
	Glycerol	50%
	SDS	4%
Γ	Orange G	0.2%

#### Acrylamide

#### 4x Running buffer

A	В
Trizma base	48 g
Glycine	230.4 g
NaN3	20 mL
ddH2O	Diluted to 4 L

#### Running buffer

A	В
4x running buffer	250 mL
ddH2O	750 mL
10% SDS	10 mL

#### Transfer buffer

А	В
4x running buffer	125 mL
ddH2O	875 mL
10% SDS	500 μL
For RABs add 20% Methanol	

- Immobilon®-FL PVDF Membrane Merck MilliporeSigma (Sigma-Aldrich) Catalog #lpfl00010
- Chameleon® Duo Pre-stained Protein Ladder LI-COR Catalog #928-60000
  - Revert™ 700 Total Protein Stain for Western Blot Normalization (250 ml)Ll-COR Catalog #926-11021

#### Revert Wash Solution

A	В
Acetic acid	6.7%
Methanol	30%
in ddH2O	

#### Revert Reversal Solution

A	В
NaOH	0.1 M
Methanol	30%
in ddH2O	

- EveryBlot Blocking Buffer 500 ml Bio-Rad Laboratories Catalog #12010020
- Primary antibodies (see Materials and Methods for specific antibodies used)
- Secondary antibodies (see Materials and Methods for specific antibodies used)

#### **Equipment**

ODYSSEY CLx Imaging System (LI-COR)

Equipment	
Mini-PROTEAN Tetra Vertical Electrophoresis Cell	NAME
Electrophoresis Cell	TYPE
Bio-Rad	BRAND
1658004	SKU
https://www.bio-rad.com/en-in/product/mini-protean-tetra-vertical-electrophoresis-cell?ID=N3F2UD4VY	LINK

Equipment	
Mini Trans-Blot Electrophoretic Transfer Cell	NAME
Electrophoretic Transfer Cell	TYPE
Bio-Rad	BRAND
1703930	SKU
https://www.bio-rad.com/en-in/sku/1703930-mini-trans-blot-electrophoretic-transfer-cell?ID=1703930	LINK



- Microcystin-LR is an extremely potent hepatotoxin and should be handled with great care.
- Acrylamide is a neurotoxin and should be handled with care.
- Methanol-containing reagents should be handled carefully, as methanol can penetrate single-layer laboratory gloves.

### **Preparation of cell lysates**

1 Quickly wash cells twice with ice-cold PBS. After the second wash, tilt the dish and completely aspirate all residual PBS.



Immediately add ice-cold lysis buffer, ensuring that the entire surface is covered by lysis buffer. Place cells on ice.

#### Note

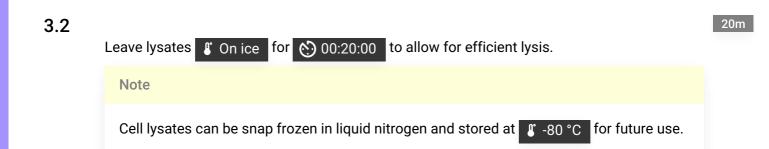
The amount of lysis buffer to use depends on cell confluency / cell number, cell type, and cell culture dish. In most cases, using  $\frac{\text{L}}{\text{L}} 100 \, \mu \text{L} = \frac{\text{L}}{\text{L}} 150 \, \mu \text{L}$  lysis buffer per well of a 6-well plate should result in a protein concentration > [M] 1  $\mu \text{g/}\mu \text{L}$ .

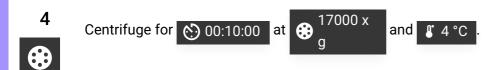
#### Note

Halt protease and phosphatase inhibitor cocktail and microcystein-LR should be added fresh on the day of use.

3 Scrape cells off the dish using a cell lifter.

3.1





10m



Discard pellet and use clarified supernatant to determine protein concentration by BCA assay following the manufacturer's instructions, performing all measurements in triplicates.

5 Add  $\underline{A}$  100  $\mu$ L  $\underline{B}$ -mercaptoethanol to  $\underline{A}$  900  $\mu$ L of 4x Protein Loading Buffer and mix well.



5.1



Add complete 4x Protein Loading Buffer to cell lysates, mix well, and boil for \$\ 00:05:00 \] at \$\ 95 \ ^C \.

Note

Do not store Protein Loading Buffer with BME for more than two weeks.

5m

### SDS-polyacrylamide gel electrophoresis

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Load samples onto 8% (for LRRK2 protein) to 15% (for PPM1H and Rab proteins) acrylamide gels alongside Chameleon Duo pre-stained protein ladder (LI-COR).



Note

Carefully rinse wells with running buffer before loading cell lysates.

7 Start electrophoresis at 80 V for 00:20:00 , then increase to 120 V and electrophorese until orange dye runs out.

20m

#### **Protein transfer**

8 Activate Immobilon-FL PVDF membrane by submerging in methanol for (5) 00:00:30



1m 30s

**(:)** 00:01:00

8.1 Wash in ddH<sub>2</sub>O and equilibrate in transfer buffer.



8.2



Soak sponges in methanol, wash in ddH<sub>2</sub>O and equilibrate in transfer buffer.

8.3 Equilibrate filter paper in transfer buffer.

8.4 Assemble blotting sandwich. 8.5

Carefully remove any air bubbles between layers using a roller.

Fill transfer tank with ice-cold transfer buffer. 9



Place transfer system § On ice 9.1

9.2

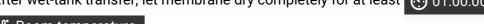


Transfer proteins from gel onto PVDV membrane at 100 V for 01:15:00

1h 15m

## Total protein stain, membrane blocking, and antibody incub

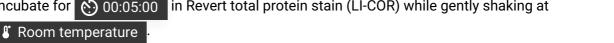
10 After wet-tank transfer, let membrane dry completely for at least 01:00:00 at



- Room temperature
- Rehydrate membrane for 00:01:00 in 100% methanol, then wash 00:05:00 in 1x TBS. 11



12 Incubate for 00:05:00 in Revert total protein stain (LI-COR) while gently shaking at



5m

12.1



Wash twice with Revert wash solution, then rinse in ddH<sub>2</sub>O and image membrane on ODYSSEY CLx imaging system.



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Rinse membrane with TBS (no detergent), then image on ODYSSEY CLx imaging system. Quantify signal intensity using Image Studio Software.