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We use this protocol and it's working

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Cell lysis and immunoblotting for protein and phospho-protein quantification

Dan Dou[1,2], C. Alexander Boecker[3], Erika L.F. Holzbaur[1,2]

[1]Department of Physiology, Perelman School of Medicine, University of Pennsylvania, Philadelphia, PA 19104, USA;
[2]Aligning Science Across Parkinson's (ASAP) Collaborative Research Network, Chevy Chase, MD, USA;
[3]Department of Neurology, University Medical Center Goettingen, 37077 Goettingen, Germany

Dan Dou

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**

	A	B
	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 **Merc MilliporeSigma (Sigma-Aldrich) Catalog #475815m**
- Pierce BCA Protein Assay Kit **Thermo Fisher Scientific Catalog #23225**

- **4x Protein Loading Buffer**

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

- Acrylamide

- **4x Running buffer**

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

- **Running buffer**

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

- **Transfer buffer**

	A	B
	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 #Ipfl00010**
- Chameleon® Duo Pre-stained Protein Ladder **LI-COR Catalog #928-60000**
- Revert™ 700 Total Protein Stain for Western Blot Normalization (250 ml) **LI-COR Catalog #926-11021**
- **Revert Wash Solution**

	A	B
	Acetic acid	6.7%
	Methanol	30%
	in ddH2O	

- **Revert Reversal Solution**

	A	B
	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

SAFETY WARNINGS

- 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.

2 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 100 µL – 150 µL lysis buffer per well of a 6-well plate should result in a protein concentration > [M] 1 µg/µL.

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

Transfer the cell lysate to an Eppendorf tube On ice .

3.2 20m

Leave lysates On ice for 00:20:00 to allow for efficient lysis.

> **Note**
>
> Cell lysates can be snap frozen in liquid nitrogen and stored at -80 °C for future use.

4 10m

Centrifuge for 00:10:00 at 17000 x g and 4 °C .

4.1

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 100 µL ß-mercaptoethanol to 900 µL of 4x Protein Loading Buffer and mix well.

5.1 5m

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.

SDS-polyacrylamide gel electrophoresis

6 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 00:00:30 - 00:01:00. 1m 30s

8.1 Wash in ddH_2O and equilibrate in transfer buffer.

8.2 Soak sponges in methanol, wash in ddH_2O 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.

9 Fill transfer tank with ice-cold transfer buffer.

9.1 Place transfer system On ice.

9.2 1h 15m

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

Total protein stain, membrane blocking, and antibody incub.. 1h 41m

10 After wet-tank transfer, let membrane dry completely for at least 01:00:00 at Room temperature. 1h

11 Rehydrate membrane for 00:01:00 in 100% methanol, then wash 00:05:00 in 1x TBS. 6m

12 Incubate for 00:05:00 in Revert total protein stain (LI-COR) while gently shaking at Room temperature. 5m

12.1

Wash twice with Revert wash solution, then rinse in ddH_2O and image membrane on ODYSSEY CLx imaging system.

13 Remove Revert total protein stain by incubating membrane in Revert reversal solution for 00:10:00 at Room temperature while gently shaking. 10m

13.1 Rinse membrane with ddH_2O.

14 Block membrane for 00:05:00 in Everyblot Blocking Buffer (Bio-Rad) at Room temperature. 5m

15 Dilute primary antibodies in Everyblot Blocking Buffer and incubate at 4 °C Overnight. 5m

16 Wash membrane in 1x TBS + 0.1% Tween-20 (TBS-T) at Room temperature (4 washes, 00:05:00 each). 5m

17 Dilute secondary antibodies 1:20,000 in Everyblot Blocking Buffer and 0.02% SDS.

Note

Incubate membrane in secondary antibodies for 01:00:00 at Room temperature.

18 Wash membrane in TBS-T at Room temperature (4 washes, 00:05:00 each). 5m

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