Cell lysis and gel electrophoresis for protein analysis of HeLa cells

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

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ABSTRACT

Here, we present multiple protocols used for biochemical analysis of protein expression and association. First, we used a simple lysis technique to determine the efficiency of an siRNA knockdown. Then, we modified two previously published methods for assaying co-precipitation of p62 and NEMO with magnetic beads conjugated to a GFP-trap molecule. In the first, we pulled down EGFP-NEMO in control or mitochondrial damaged conditions, and in the second, we pulled down EGFP-Ubiquitin in p62-/- cells with expression of wild-type p62 or a dysfunctional mutant. Since p62 is known to form multimers, we used specialized buffers to preserve those putative interactions. We were able to reproduce results published previously by pulling down EGFP-Ubiquitin in p62-expressing cells. However, interestingly, we did not find evidence that NEMO interacts with p62 in the soluble fraction, or via ubiquitin chains generated in basal conditions. These studies demonstrated that NEMO recruitment to damaged mitochondria occurs in specific circumstances, and NEMO colocalization with p62 is also dependent on multiple factors.

ATTACHMENTS

470-986.pdf

GUIDELINES

- This protocol was developed to analyze protein expression and enrichment in cell culture, including HeLa-M cells and HeLa p62-/- cells.
- Option 2 was modified from a protocol used in Turco et al, Molec. Cell, 2019.
- Option 3 was modificed from a protocol used in Wurzer et al, eLife, 2015.

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Keywords: Cell culture, Cell lysis, Immuno-precipitation, Gel electrophoresis, Western blotting, ASAPCRN

MATERIALS

Materials

- 1.5 mL capped tubes **Merck MilliporeSigma (Sigma-Aldrich) Catalog #EP022364120**
- Cell scrapers
- Liquid nitrogen
- Protein LoBind tubes **Eppendorf Catalog #022431081**
- 10% acrylamide gels with desired number of wells (make or purchase)

Reagents:

For all Options:

- 1X Phosphate buffered saline (PBS)
- Fisher BioReagents™ Bovine Serum Albumin Fraction V Cold-ethanol Precipitated **Fisher Scientific Catalog # BP1605100**

For Option 2:

- 10% sodium dodecyl sulfate solution (SDS) **Thermo Fisher Scientific Catalog #15553035**
- **Protease and phosphatase inhibitors**

	A	B	C
	Leupeptin	1000 X	10 mg/mL
	DTT	1000 X	1M
	Pepstatin A	1000 X	1 mg/mL
	TAME	1000 X	10 mg/mL
	PMSF	100 X	100 mM

- **4X Denaturing buffer (DB) (900uL)**

	A	B

	A	B
	SDS	4%
	Glycerol	50%
	Tris HCl, pH 6.8	125 mM
	Orange G	0.2% w/v
	Betamercaptoethanol (BME)	100 uL

- Methanol
- 4X Running buffer (RB)
- 1X Tris buffered saline (TBS)
- 1X TBS with 0.1% Tween (
  Tween 20 100% Nonionic Detergent **Bio-Rad Laboratories Catalog #1706531**
) (TBST)

Running Buffer (RB):

	A	B
	4X RB	250 mL
	Water	750 mL
	10% SDS	10 mL

Transfer buffer:

	A	B
	4X RB	125 mL
	Water	775 mL
	Methanol	100 mL
	10% SDS	500 uL
	Betamercaptoethanol (BME)	560 uL

- PVDF membranes
- Revert™ 700 Total Protein Stain for Western Blot Normalization (250 ml) **LI-COR Catalog #926-11021**

- **REVERT Wash Buffer:**

	A	B
	Glacial acetic acid	6.7% w/v
	Methanol in water	30% v/v

- **REVERT Reversal Buffer:**

	A	B
	NaOH	0.1 M
	Methanol in water	30% v/v

- Desired primary antibodies
- LICOR secondary antibodies such as
  IRDye® 800CW Donkey anti-Mouse IgG Secondary Antibody **LI-COR Catalog #926-32212**
  and
  IRDye® 680RD Donkey anti-Rabbit IgG Secondary Antibody **LI-COR Catalog #926-68073**
- TrueBlack buffer (Biotium 23013B-1L) **Biotium Catalog #23013B-1L**
- EveryBlot Blocking Buffer 500 ml **Bio-Rad Laboratories Catalog #12010020**

Specialized buffers and other reagents:

RIPA buffer (Option 1)

	A	B	C	D
	Reagent	Stock concentration	Final concentration	Volume of stock (for 10 mL)
	Tris-HCl (pH 8.0)	1 M	50 mM	500 uL
	EDTA	500 mM	1 mM	20 uL
	EGTA	200 mM	2 mM	100 uL
	Triton X-100	10 %	1 %	1000 uL
	DOC	5 %	0.50 %	1000 uL

	A	B	C	D
	SDS	10 %	0.10 %	100 uL
	NaCl	5 M	150 mM	300 uL
	Water	-	-	7 mL

Lysis Buffer-A (Option 2):

	A	B	C
	Reagent	Final conc	For 10 mL
	1 M HEPES/KOH	50 mM pH 7.5	500 uL
	1 M Sorbitol	250 mM	2.5 mL
	200 mM EGTA	0.5 mM	25 uL
	1 M Mg-Acetate	5 mM	50 uL
	ddH2O	-	6.92 mL

- PBS with 0.1% TWEEN (PBST) for Option 2

Wash Buffer (Option 3):

	A	B	C
	Reagent	Final conc	For 20 mL
	1 M Tris-Cl	20 mM, pH 7.4	400 uL
	100% Glycerol	10%	2 mL
	5 M NaCl	135 mM	540 uL
	ddH2O	-	17.06 mL

Lysis Buffer-B (Option 3):

	A	B	C
	Reagent	Final conc	For 5 mL
	Master buffer	20 mM, pH 8.0	4.975 mL
	100% NP-40 (IGEPAL)	0.5%	25 uL

- Pierce BCA Protein Assay Kit **Thermo Fisher Scientific Catalog #23225** for Option 1
- ChromoTek Spot-Trap® Magnetic Particles M-270 **ChromoTek Catalog #M-270**

Equipment:

- Vacuum apparatus
- End-on-end rotating apparatus
- Refrigerated centrifuge
- Magnetic rack (for GFP-Trap particle precipitation)
- Rockers at Room temperature and at 4 °C
- Plate reader (such as BioTex Synergy Mx)

Equipment	
Odyssey® DLx Imaging System	NAME
Imaging System	TYPE
Licor	BRAND
LI-COR, 9140	SKU
https://www.licor.com/bio/odyssey-dlx/	LINK

- ImageStudio software (LI-COR)
- Heat source to 95 °C
- Gel electrophoresis apparatus (BIO-RAD)
- Membrane transfer apparatus (BIO-RAD)
- Excel

BEFORE START INSTRUCTIONS

- The start point for this protocol is after cells grown on 3.5 cm, or 10cm dishes have been transfected with relevant constructs for 18:00:00 - 24:00:00 and treated with appropriate small molecules or vehicles.
- For 3.5 cm dishes, follow transfection procedures enumerated in imaging protocols.
- Chill all reagents On ice.
- Add protease and phosphatase inhibitors to 1X to each lysis buffer immediately before use.

Wash cells

1 Aspirate media from dishes.

2 Wash samples quickly x2 with ice cold PBS.

Note

Can stop after washes by scraping cells with the second wash of PBS into 1.5 mL tube, spin down at 2400 x g, 00:03:00, 4 °C. Aspirate PBS and snap-freeze tubes in liquid nitrogen. If frozen, add respective lysis buffer and inhibitors and let thaw On ice 00:10:00 before proceeding.

Step 2 includes a Step case.
Standard lysis with RIPA
Co-IP EGFP-NEMO for p62 association assay
Co-IP GFP-Ubiquitin for p62 and NEMO

step case

Standard lysis with RIPA

3

Note

We used this protocol to assess depletion of p62 in HeLa-M cells after siRNA treatment and imaging NEMO recruitment. Samples were collected from 35 mm imaging dishes.

Add 150 µL RIPA + inhibitors to dish and scrape cells into 1.5 uL tube, OR add buffer to thawed sample and resuspend by pipetting.

4 Rotate resuspended sample on end-over-end machine at 4 °C for 00:20:00. 20m

5 Spin at top speed (17000 x g), 4 °C, 00:20:00. 20m

6 Remove supernatant as Lysis and keep On ice or store at -80 °C.

7 Measure protein concentration with Pierce BCA assay by adding 25 µL sample or BSA standard to each well in duplicate and 200 µL Reagent A+B. Incubate 37 °C for 00:30:00 then measure absorbance on a plate reader. 30m

Note

It is likely necessary to dilute samples 1:4 or more to measure within the range of the assay.

8 Add 1/3 volume of 4X DB to remaining Lysis or a measured fraction of sample and heat 95 °C for 00:05:00. 5m

9 Proceed to gel electrophoresis.

Gel electrophoresis and immune-blotting

10

Note

This protocol was developed for use with the LI-COR system for protein detection.

Gel electrophoresis and immune-blotting: Set-up

11 Set up electrophoresis cell with 10% gels by manufacturer's instructions.

12 Fill cell with RB and flush wells with a plastic transfer pipet.

13 Invert samples by hand to mix, and ensure all samples are at the bottom of tubes by briefly centrifuging.

14 Load wells with equal amounts of protein (Option 1) or equal volumes (Options 2 and 3) and molecular weight standard (4 µL - 5 µL).

Note

We load 15 µL - 25 µL eluate and 10 µL Input.

15 For empty lanes, load approx. equal volume of 1X DB.

Gel electrophoresis and immune-blotting: Running

16 Run samples through stacking gel (85 V, 00:20:00 - 00:40:00). 1h

17 Run samples through 10% gel (125 V, until front has reached bottom of gel, usually ~ 01:10:00). 1h 10m

Gel electrophoresis and immune-blotting: Transfer

18 Remove gels from electrophoresis cell and construct transfer cassettes with PVDF membranes according to manufacturer's instructions.

19 Place the cassettes in the transfer cell and fill cell with Transfer buffer and icepack.

20 Place the cell in a basin.

21 Fill basin with ice around cell.

22 Run transfer for 01:00:00 - 01:10:00, 100 V. 2h 10m

Gel electrophoresis and immune-blotting: Membrane proce...

23 45m

Dry membrane between filter paper in the dark for at least 00:45:00.

Note

- This is most important for small proteins.
- Can be a stopping point for several days.

24 Rehydrate membrane in MetOH.

25 Wash in ddWater.

26 Wash in 1X TBS 00:02:00. 2m

27 Stain total protein, 00:05:00, Room temperature, with REVERT 700 Total Protein Stain. 5m

28 Wash membrane with REVERT wash buffer.

28.1 Wash membrane 2x 00:00:30 with REVERT wash buffer (1/2). 30s

28.2 Wash membrane 2x 00:00:30 with REVERT wash buffer (2/2). 30s

29 Image total protein on LICOR.

Note

Can cut the membrane based on total stain if desired.

30 Wash off total stain with REVERT Reversal (up to 00:10:00, Room temperature) 10m

31 Rinse in ddWater.

Gel electrophoresis and immune-blotting: Immuno-labeling

32 Block membranes in EveryBlot buffer, 00:05:00, Room temperature with rocking. 5m

33 Incubate in vacuum packs with primary antibodies in EveryBlot Overnight at 4 °C. 5m

Note

See materials and methods for concentrations of antibodies used.

34 Wash with TBST.

34.1 Wash with TBST 4x 00:05:00 (1/4). 5m

34.2 Wash with TBST 4x 00:05:00 (2/4). 5m

34.3 Wash with TBST 4x 00:05:00 (3/4). 5m

34.4 Wash with TBST 4x 00:05:00 (4/4). 5m

35 Incubate with secondary antibody 1:20,000 in TrueBlack antibody diluent with 0.2% TWEEN and 1:1000 10% SDS for up to 01:00:00. 1h

36 Wash with TBST.

36.1 Wash with TBST 4x 00:05:00 (1/4). 5m

36.2 Wash with TBST 4x 00:05:00 (2/4). 5m

36.3 Wash with TBST 4x 00:05:00 (3/4). 5m

36.4 Wash with TBST 4x 00:05:00 (4/4). 5m

37 Wash 1X with TBS to clear TWEEN.

38 Wash 1X with water.

39 Image

Gel electrophoresis and immune-blotting: Quantification

40

For quantification of knockdown (Option 1).

40.1 Use ImageStudio software to draw rectangles around total protein in each lane of Total Protein image and subtract
background.

40.2 Then add rectangles to outline p62 bands and subtract background.

40.3 Transfer intensity measurements to Excel.

40.4 Calculate p62 expression relative to total protein for each experiment.

41 For quantification of p62 enrichment from GFP-NEMO immunoprecipitation.

41.1 Use ImageStudio to add rectangles around input/cytosolic p62 and eluted p62.

41.2 Transfer intensity measurements with background subtracted to Excel.

41.3 Calculate p62 eluted relative to input amounts.

41.4 Calculate elution/input quantity relative one condition.

Note

We found it necessary to perform this secondary normalization due to variability across replicates. In our case, we normalized results from each condition to the EGFP-NEMO + AntA/OligA results.