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Optimized automated capillary Western blotting method for Miro1 detection (Layla Drwesh)

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Layla Drwesh[1]

[1]Hertie Institute for Clinical Brain Research

Julia Fitzgerald
Hertie Institute for Clinical Brain Research

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We use this protocol and it's working

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Abstract

Here we describe an optimized assay for the detection and quantification of Miro1 protein using automated capillary Western blotting. We also describe how to multiplex Miro1, mitofusin and other marker controls for mitophagy induction following mitochondrial depolarization.
We describe the conditions for culture, seeding and treatment of fibroblasts, PBMCs and human dopaminergic neurons and describe a detailed protocol for preparation of the cell lysates and for performing capillary Western blotting.

Protocol description

1 Detecting the Miro1 signal using traditional Western blotting presented several challenges, the relatively low abundance of Miro1 in fibroblasts combined with the low sensitivity of available antibodies, and variations in Western blotting procedures, lead to reproducibility issues. To overcome these challenges, we optimized a standardized approach using the fully automated JESS Simple Western™ system (ProteinSimple®, Bio-Techne), which allows for both quantitative and qualitative analysis. This capillary Western blot method automates all steps involved in traditional Western blotting, from protein size separation to immunoblotting and signal quantification, however optimizing assay conditions—such as protein concentration, antibody dilution, and exposure time—is crucial for ensuring accurate and reproducible results.
The process used to optimize protein concentrations, antibody dilutions, and exposure times for an automated capillary-based size assay is demonstrated using whole cell extracts isolated from Fibroblasts,
Peripheral blood mononuclear cells (PBMCs), and dopaminergic neurons (DANs).
Samples total protein concentrations should be determined beforehand using established methodologies compatible with the lysis buffer used. In our study, we have used RIPA lysis buffer ([M] 100 millimolar (mM) Tr is, 150 mM NaCl, 1 mM EGTA, 1 mM EDTA, 1% Triton X-100, 0.5% sodium deoxycholate, 1mM PMSF, 1:10 protease inhibitor cocktail (539134, Calbiochem, San Diego, United States of America). And protein estimation was done using Pierce BCA Protein Assay Kit (Thermofisher, #23225).

Equipment

2 1. Benchtop centrifuge

2. Microcentrifuge tubes (1.5 mL)

3. Thermomixer

4. Capillary Western blotting machine (in this case: JESS Simple Western™ system, Bio-Techne).

List of materials:

3 1. EZ standard pack I (#PS-ST01EZ-8)

2. 12–230 kDa separation module, 8 × 25 capillary cartridge (#SM-FL004 ProteinSimple®, Bio-Techne)

3. Ready to use goat anti-rabbit secondary HRP-conjugated antibody (#042-206, ProteinSimple®).

4. Ready to use goat anti-mouse secondary HRP-conjugated antibody (#042-205, ProteinSimple®)

5. ECL reagents (S-luminol and peroxide) (#DM-001, ProteinSimple®)

6. Milk-free antibody diluent buffer 2 (#042-203, Bio-Techne).

7. Replex module (#RP-001)

Reagents preparation from the standard pack supplied by the manufacturer

4 *Note: all the three tubes are sealed by the manufacturer with a foil cover and must be pierced by a pipette tip.*

5 Add 40 µL dH_2O to the tube containing dithiothreitol (DTT) To prepare 400 mM working solution. Mix
the solution gently to avoid producing bubbles

6 Add 20 µL 10X sample buffer and 20 µL of the prepared 400 mM DTT solution to the pink tube containing fluorescent 5X master mix. Mix gently.

7 Add 20 µL dH2O to the white biotinylated ladder tube. Mix gently

Working with human fibroblasts

8 Fibroblasts were seeded from cryovials and
maintained in a 37^0C, 5% CO2 incubator with humidified atmosphere and cultured in high glucose (4.5 g/L) DMEM (Sigma #D6429) containing 10% fetal bovine serum, supplemented with 1% non-essential amino acids (Thermofisher #11140035) and 1% penicillin-Streptomycin (Sigma-Aldrich #P0781). The plates were regularly examined, and the medium was replaced every 3–4 days until fibroblasts reached confluency. Confluent fibroblasts were trypsinized and transferred to a flask for further growth, with subsequent expansion into

multiple dishes or flasks at a 1:4 split ratio. For storage, batches of ~2.5 x 10^5 cells per 0.5 ml of growth medium with 10% DMSO were frozen in cryo-vials. The temperature was gradually decreased before storage in liquid nitrogen by placing the vials in a freeing box containing isopropanol in a -80 °C freezer overnight up to 1 weak before placing in liquid nitrogen container for long term storage.

Working with PBMCs

9 Immediately following blood draw and collection in three BD Vacutainer CPT^{TM} vials, the vials containing around 8-10 mL of blood were processed in the laboratory within 2 hours of the blood draw. The vials are inverted gently 8-10 times and centrifuged at 1,700-1500 × g for 20 min at room temperature in a swinging-bucket rotor. After centrifugation, the vials are inverted again 8-10 times. The plasma supernatant containing PBMCs was transferred
into new 50 mL tube, washed once with PBS, and centrifuged at 300 × g for 5 minutes. The supernatant was carefully aspirated and the pellet containing PBMCs and remaining red blood cells (RBCs) was resuspended with 5-7 mL Erylyse buffer (0.1 mM EDTA, 155mM NH_4Cl, 10mM $KHCO_3$, pH 7.4) and
incubated for 5 minutes to lyse RBCs, and then washed with PBS and centrifuged at 300 × g for 5 minutes. Cells were resuspended in 90% fetal bovine serum (FBS) + 10% DMSO at -80^{0}C, followed by liquid nitrogen storage 24h later.
Cell viability and concentration were assessed by Trypan Blue using TC10 automated cell counter (Biorad). Where possible, 4-5×10^6. cells were stored per cryovial.

Working with human dopaminergic neurons

10 NPCs were cultures in NPC maintainance medium,
containing; base medium; (50% DMEM/F12 (Thermo Fisher Scientific (Waltham, MA, USA), #11-330-057), 50% neurobasal (Thermo Fisher Scientific (Waltham, MA, USA), 21103-049), 1% penicillin/streptomycin (Merck (Darmstadt, Germany), #A2213), 1% GlutaMax (Thermo Fisher Scientific (Waltham, MA, USA), #35050-038), 1% B27 supplement (without vitamin A; Thermo Fisher Scientific (Waltham, MA, USA), #12587-010), and 0.5% N2 supplement (Thermo Fisher Scientific (Waltham, MA, USA), #17502-048)) supplemented with 3 µM CHIR 99021, 200 µM ascorbic acid (Sigma-Aldrich (St. Louis, MO, USA), #A4544-25G), and 0.5 µM PMA) and plated on Matrigel (Corning (Corning, NY, USA), #354230). After several passages, NPCs were differentiated into dopaminergic neurons using small molecules as previously described
(Reinhardt et al. 2013).

Mitochondrial depolarization

11 **Fibroblasts:**
Each cell line was split to three wells in either 6 or 12 well-plate at a similar density prior to treatment. CCCP (C2759, Sigma-Aldrich) was prepared at 40 mM in DMSO and small volume aliquots were stored in -80 ^{0}C. 40 µM final CCCP concentration was applied to cell lines in fresh culture medium (high glucose DMEM, 10% fetal bovine serum, 1% non-essential amino acids and 1% penicillin-Streptomycin) at 1:1,000 dilution. The wells were treated as follows: first well: treated with DMSO (vehicle
control), second well: treated with 40 µM of CCCP for six hours to induce mitophagy by depolarization and third well: treated with 10 µM of the proteasome inhibitor MG132 in addition to 40 µM CCCP.

PBMCs:
Human PBMCs were thawed in a 37°C water bath, transferred to 15 mL of RPMI-1640, and centrifuged at 300 x g. The cell pellet was resuspended in RPMI-1640 with 10% FBS, 1% penicillin-streptomycin, and
1:1000 Apoi, then seeded in a non-coated Petri dish and incubated overnight. The next day, cells were collected, centrifuged, and seeded in 6-well plates (3-4 million cells per well). After 2 hours, cells were treated with 10 µM Oligomycin and 10 µM Antimycin in one well, and EtOH vehicle control in the
other, for 6 hours. After treatment, cells were collected, washed with PBS, centrifuged, and either stored at -20°C or immediately lysed in 20 µL lysis buffer (100 mM Tris, 150 mM NaCl, 1 mM EGTA, 1% Triton X-100, 0.5% sodium deoxycholate, 1 mM PMSF, 1:10 protease inhibitor cocktail).

hDaNs:
Dopaminergic neurons at day 16–19 were treated for 0, 2, 4, 6, or 24 h with a final concentration of 100 µM Antimycin A in the neuronal maturation medium. Untreated hDaNs received fresh neuronal maturation medium with EtOH (vehicle control) for 24 h.

Preparation of lysates

12 Following treatment, cells were collected from the well by; trypsinization (fibroblasts), collection (PBMCs) and Accutase (hDaNs), and washed once with PBS, followed by centrifugation for 5 minutes at 300 x g. The cell pellet was then resuspended with 20-50 µl lysis buffer:

(100 mM Tris, 150 mM NaCl, 1 mM EGTA, 1 mM EDTA, 1% Triton X-100, 0.5% sodium deoxycholate, 1mM PMSF, 1:10 protease inhibitor cocktail (539134), Calbiochem),

and was either stored in -80 ^{0}C until further treatment or proceeded with 15-30 minutes incubation on ice to enable cell lysing. Supernatant of Whole cell lysates (WCL) were then collected after centrifugation for 15-30 minutes at 18,000 x g 4 ^{0}C. Protein estimation was performed using Pierce BCA Protein Assay Kit (Thermofisher,#23225). All lysates were diluted using lysis buffer to have
the same final concentration (typically between 1-2 mg/mL).

Sample preparation for capillary Western blotting

13 Calculate the amount of 0.1x sample buffer that is added to a sample, which will depend on the final desired concentration of the total protein.

14 Mix 1 part of 5x fluorescent master mix with 4 parts of diluted sample to achieve the desired final protein concentration

15 Calculation was done as follows:
(i) Volume 5x fluorescent master mix = (Desired final protein concentration)/5
(ii) Volume of protein stock = (Desired final protein concentration x Total volume needed)/ Protein stock concentration
(iii) Volume 0.1x sample buffer = Total volume - 5x master mix volume - Protein stock volume.

16 Denaturation of samples: Place prepared samples in a 95 °C heat block for 5 minutes. Vortex tubes immediately after incubation, spin for ~5 s in a tabletop centrifuge, and place On ice .

Antibodies preparation

17 Generally, antibodies dilutions used in capillary based WB are 100 x higher concentrated compared to the dilution used in traditional western blotting.

18 Antibodies used for this assay are listed below and diluted with milk-free antibody diluent buffer:
1. Miro1 (#HPA01687, Merck) at 1:10 dilution
2. Mfn2 (#H00009927, Abnova) at 1:50 dilution
3. GAPDH (#CB1001, Merck) at 1:1,000
4. VDAC (#AB10527, millipore) at 1:1,000