Dec 03, 2024

Protein Lysate and Immunoblotting

The Journal of Cell Biology

DOI
dx.doi.org/10.17504/protocols.io.5qpvo9bmdv4o/v1

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DOI: https://dx.doi.org/10.17504/protocols.io.5qpvo9bmdv4o/v1

Protocol Citation: Amanda Bentley-DeSousa, Shawn Ferguson 2024. Protein Lysate and Immunoblotting. **protocols.io https://dx.doi.org/10.17504/protocols.io.5qpvo9bmdv4o/v1**

Manuscript citation:
https://doi.org/10.1101/2023.10.31.564602

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

Created: December 02, 2024

Last Modified: December 03, 2024

Protocol Integer ID: 113416

Keywords: RAW 264.7, protein lysate, immunoblotting, lysate, protein, protocol details step

Funders Acknowledgements:
Aligning Science Across Parkinson's Disease
Grant ID: ASAP-000580

Abstract

This protocol details steps taken to create protein lysates and perform immunoblotting in RAW 264.7 cells.

Materials

DMEM (Thermo Fisher Scientific, 11965-092)
FBS (Thermo Fisher Scientific, 16140-071)
Penicillin/Streptomycin (Thermo Fisher Scientific, 15140122)
CellStripper (Corning, 356230)
PBS [1.1 mM KH2PO4 (J.T. Baker, 3246-01), 155.2 mM NaCl (Sigma-Aldrich, 3624-05), and 3 mM Na2HPO4 (J.T. Baker, 3828-05)]
Lysis Buffer [50 mM Tris Base (American Bio, AB02000-05000), 150 mM NaCl (Sigma-Aldrich, 3624-05), 1% Triton X-100 (Sigma-Aldrich, X100), 1 mM EDTA (Sigma-Aldrich, 03690) supplemented with cOmplete mini EDTA-free protease inhibitor (Roche, 11836170001) and PhosSTOP (Roche, 4906837001)
Coomassie Plus Protein Assay Reagent (ThermoFisher Scientific, 23236)
Laemmli Buffer [80 mM Tris-HCl pH 6.8 (American Bio, AB020000-05000 / HCl, Sigma-Aldrich, 3624-06), 25.3% Glycerol (American Bio, AB00751), 2.67% SDS (American Bioanalytical, AB01920-00500), and Bromphenol Blue (Sigma-Aldrich, B5525) supplemented with 6.187% fresh b-mercaptoethanol (Sigma-Aldrich, M3148)]
4-15% miniPROTEAN TGX Stain-Free pre-cast gels (BioRad, 4568084/4568085/4568086)
Electrophoresis Buffer [24.76 mM Tris Base (American Bio AB02000-05000), 191.87 mM Glycine (American Bio AB00730-05000), 10 mL 10% SDS (American Bio, AB01920-00500)]
0.45 µm Pore Nitrocellulose Membrane (Thermo Fisher Scientific, 1620115)
Transfer Buffer [24.76 mM Tris Base (American Bio AB02000-05000), 191.87 mM Glycine (American Bio AB00730-05000), 20% Methanol (Sigma-Aldrich, 179337-4l-pB)]
Non-fat dry milk omniblock (American Bio, AB 10109-01000)
TBST [10 mM Tris Base (American Bio AB02000-05000), 150 mM NaCl (Sigma-Aldrich, 3624-05), 0.1% Tween 20 (Sigma-Aldrich, P7949)]
BSA (Sigma-Aldrich, A9647)
SuperSignal West Pico PLUS Chemiluminescence Substrate (Thermo Fisher Scientific, 34580)
SuperSignal West Femto Maximum Sensitivity Substrate (Thermo Fisher Scientific, 34095)

Troubleshooting

Day 0

1 Plate 1 × 10^6 RAW 264.7 cells per well in a 6-well dish.

Day 1

1h 41m

2 Add the indicated compound at the provided concentration and time point in fresh DMEM media containing FBS and P/S.

2.1 Proceed directly to the next step if not treating cells.

3 Wash each dish 2X with cold PBS on ice.

4 Remove the PBS, scrape each well with 50 µL of ice-cold lysis buffer, and transfer the lysates to Eppendorf tubes.

4.1 Lysis Buffer: 50 mM Tris Base, 150 mM NaCl, 1% Triton X-100, and 1 mM EDTA supplemented with cOmplete mini EDTA-free protease inhibitor and PhosSTOP.

5 Incubate on ice for 00:05:00 .

6 Centrifuge 14000 rpm, 4°C, 00:08:00 lysates. 8m

7 Measure the protein concentration of the supernatant using Coomassie Plus Protein Assay Reagent as per the manufacturer's instructions.

8 Mix the supernatant/lysate 1:1 with Laemmli Buffer and heat at 95 °C for 00:03:00 . 3m

8.1 Laemmli Buffer: 80 mM Tris-HCl pH 6.8, 25.4% Glycerol, 2.67% SDS, and Bromophenol Blue
supplemented with 6.187% fresh B-mercaptoethanol.

9 Perform electrophoresis using between 20-30 uG of protein in 4-5% miniPROTEAN TGS Stain-Free pre-cast gels.

9.1 Electrophoresis Buffer: 24.76 mM Tris Base, 191.87 mM Glycine, and 1% SDS.

10 After electrophoresis, transfer gels onto 0.45 um pore nitrocellulose membrane at 100V for 01:00:00 . 1h

10.1 Transfer Buffer: 24.76 mM Tris Base, 191.87 mM Glycine, and 20% methanol.

11 Block membranes in 5% non-fat dry milk omniblock in TBST for 00:30:00 . 30m

11.1 TBST: 10 mM Tris Base, 150 mM NaCl, 0.1% Tween 20.

12 Add antibodies in 5% BSA TBST 4 °C Overnight at the indicated concentrations.

Day 2 1h 20m

13 Wash membranes 2X 00:10:00 with TBST. 10m

14 Add secondary antibodies in either 5% milk TBST (phospho antibodies) or TBST for 01:00:00 at Room temperature . 1h

15 Wash membranes 3X 00:10:00 with TBST. 10m

16 Develop membranes via chemiluminescence (SuperSignal West Pico PLUS Chemiluminescence Substrate or SuperSignal West Femto Maximum Sensitivity Substrate) and image with a Biorad Chemidoc MP Imaging Station.