Immunoblotting

Authors: Will Hancock-Cerutti[1,2,3], Pietro De Camilli[1,3]

[1]Departments of Neuroscience and of Cell Biology, Howard Hughes Medical Institute, Program in Cellular Neuroscience, Neurodegeneration and Repair, Yale University School of Medicine, New Haven, Connecticut 06510, USA

[2]Interdisciplinary Neuroscience Program and MD-PhD Program, Yale University School of Medicine, New Haven, Connecticut 06510, USA

[3]Aligning Science Across Parkinson's (ASAP) Collaborative Research Network, Chevy Chase, MD, 20815

Abstract

This protocol describes collection of protein from cultured cells and immunoblotting, including immunoblotting of large proteins using a proprietary tris-acetate buffer system.

Keywords

Immunoblot, Western blot, tris-glycine, tris-acetate, siRNA, cGAMP, HT-DNA

Solutions to prepare

DMEM containing 10% FBS, 100 U/ml penicillin, 100 mg/ml streptomycin, and 2 mM L-glutamine (all from Gibco).
RIPA buffer: 150 mM NaCl, 10 mM Tris, 0.5 mM EDTA, 0.5% NP40 supplemented immediately before use with Protease Inhibitor Cocktail (Roche) and PhosStop phosphatase inhibitor (Roche)
TBS: 50 mM Tris-Cl, 150 mM NaCl, adjust pH to 7.5
TBST: TBS with 0.1% TWEEN-20 (Sigma-Aldrich)
3x Laemmli buffer: 188 mM Tris-HCl, 3% SDS, 30% glycerol, 0.01% bromophenol blue, 15% β-mercaptoethanol
Tris-glycine running buffer: 25 mM tris base, 192 mM glycine, 0.1% SDS in milliQ water
Tris-glycine transfer buffer: 25 mM Tris, 192 mM glycine, 20% methanol in milliQ water. Chill to 4 ˚C.

A. Cell culture and treatments

1. HeLa-M cells were cultured at 37°C in 5% CO_2 and DMEM containing 10% FBS, 100 U/ml penicillin, 100 mg/ml streptomycin, and 2 mM L-glutamine (all from Gibco).
2. For any given experiment, cells were plated at such density so as to be approximately 90% confluent at the time of lysis.
3. For experiments using siRNA, 60 pmols of the indicated siRNA was transfected using 6μL Lipofectamine RNAiMax (ThermoFisher) in Opti-MEM (Gibco) per well according to manufacturer protocol. Cells were lysed 72 hours after siRNA transfection.
4. For experiments using cGAMP, 8 μg/L of cGAMP was transfected using using 6μL Lipofectamine RNAiMax (ThermoFisher) in Opti-MEM (Gibco) per well according to manufacturer protocol.
5. For experiments using herring testes (HT)-DNA, 1 ug HT-DNA was transfected using using 3 μL Lipofectamine 2000 (ThermoFisher) in Opti-MEM (Gibco) per well according to manufacturer protocol.

B. Cell lysis and sample preparation

1. Supplement RIPA buffer with Protease Inhibitor Cocktail (Roche) and PhosStop phosphatase inhibitor (Roche) and chill on ice.
2. Aspirate media from cells and rinse cells with PBS on ice. Aspirate PBS thoroughly.
3. Pipette RIPA lysis buffer onto cells and scrape cells using a cell lifter (Corning).
4. Pipette lysis buffer containing cell mass into Eppendorf tube.
5. Incubate Eppendorf tube on ice for 30 minutes.
6. Every 10 minutes, pipette lysis mixture up and down 10 times with a P-200 pipette tip (a total of 3 cycles).

NOTE: Take care not to introduce bubbles.

7. Centrifuge at 15,000 x *g* for 10 min at 4˚C and collect the post-nuclear supernatant in a new Eppendorf tube.

NOTE: Samples can be snap frozen in liquid nitrogen at this step and stored at -70˚C.

8. Determine protein concentration in sample using Pierce BCA assay (ThermoFisher)
9. Prepare samples at desired concentration and add 3x Laemmli buffer.

C. Gel electrophoresis and immunoblotting (Tris-glycine buffer system)

1. Incubate samples at 95˚C for 5 minutes.
2. During this incubation, prepare gel apparatus with Mini PROTEAN TGX 4-20% tris-glycine gels (Bio-Rad) and running buffer.
3. Load samples into gel and run until dye front reaches bottom (90-120 V).
4. Remove gel and set up transfer cassette with nitrocellulose membrane.
5. Transfer at 120 V for 1 hour at 4˚C in tris-glycine transfer buffer.
6. Remove nitrocellulose membrane and stain for total protein with ponceau stain.
7. Wash with milliQ water.
8. Block membrane with 5% milk in TBST for 1 hour at 22˚C.
9. Incubate membrane with primary antibodies in 2.5% milk in TBST overnight at 4˚C .

NOTE: Optimal primary antibody incubation time and temperature can be determined empirically for a given primary antibody

10. Wash membrane for 5 minutes with TBST. Repeat a total of 3 times.
11. Incubate membrane with secondary antibodies conjugated to IRdye 800CW or IRdye 680CW (1:10,000, Licor) in 2.5% milk in TBST for 1 hour at 22˚C
12. Wash membrane for 5 minutes with TBST. Repeat a total of 3 times.
13. Wash membrane for 5 minutes with TBS. Repeat a total of 3 times.
14. Image membranes using a Licor Odyssey Infrared Imager.

D. Gel electrophoresis and immunoblotting (Tris-acetate buffer system)

1. For VPS13C immunoblotting, samples were lysed and post-nuclear supernatant was collected as above.
2. Mix post-nuclear supernatant with NuPAGE LDS Sample Buffer and Reducing Agent (Thermofisher) and incubated for 10 minutes at 70 ˚C.
3. During this incubation, prepare gel apparatus with NuPage Tris-Acetate 3-8% gels and NuPage Running Buffer (Thermofisher)
4. Remove gel and set up transfer cassette with nitrocellulose membrane.
5. Transfer at 0.05 mA for 16 hours at 4˚C in NuPage transfer buffer (Thermofisher).
6. Remove nitrocellulose membrane and stain for total protein with ponceau stain.
7. Wash with milliQ water.
8. Block membrane with 5% milk in TBST for 1 hour at 22˚C.
9. Incubate membrane with primary antibodies in 2.5% milk in TBST for 2 hours at 22˚C.

NOTE: Optimal primary antibody incubation time and temperature can be determined empirically for a given primary antibody

10. Wash membrane for 5 minutes with TBST. Repeat a total of 3 times.
11. Incubate membrane with secondary antibodies conjugated to IRdye 800CW or IRdye 680CW (1:10,000, Licor) in 2.5% milk in TBST for 1 hour at 22˚C.
12. Wash membrane for 5 minutes with TBST. Repeat a total of 3 times.
13. Wash membrane for 5 minutes with TBS. Repeat a total of 3 times.
14. Image membranes using a Licor Odyssey Infrared Imager.