STANDARD OPERATING PROCEDURE

Title: Evaluation using Western Blot

SOP#: M-103

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1. PURPOSE

The purpose of this document is to describe the procedure for performing Western Blot analysis for detection of specific proteins, either recombinant or from a sample of tissue homogenate or cellular extract.

2. SCOPE

This procedure may be used for proteins with molecular weights in the range of 10KDa to 250KDa. Other procedures must be used for proteins outside of this range.

3. RESPONSIBILITIES

It is the responsibility of person(s) performing this procedure to be familiar with lab safety procedures. The interpretation of results must be done by a person trained in the procedure and familiar with such interpretation.

4. EQUIPMENT

- Trans-Blot Turbo Transfer System – Bio-Rad Cat# 1704150
- Labcor™ Mini Western Blot staining trays, 35x86x12.7mm; Continental Lab Products Cat. #731-002 or equivalent
- Magnetic stir plate
- Rocking platform; LabLine model 4831 or equivalent
- Water bath or heating block with appropriate insert pre-heated to 95°C
- ChemiDoc MP Imaging System (Bio-Rad Cat # 12003154) or equivalent
- Precision pipettes

5. MATERIALS

- Pre-cast, 4–20% Criterion™ TGX Stain-Free™ Protein Gel, 18 well, 30 µl Bio-Rad Cat. #5678094
- One-dimension SDS-PAGE running buffer: 10x Tris/Glycine/SDS Buffer diluted to 1x with deionized water; Bio-Rad Cat. #1610732
- Protein molecular weight standards: Precision Plus Protein™ All Blue Standards; Bio-Rad Cat. #1610373
- Opti-4CN Substrate Kit – Bio-Rad Cat. #1708235
- Clarity Western ECL Substrate – Bio-Rad Cat. #1705061
- Phosphate Buffered Saline (PBS), 10x solution (Fisher Scientific Cat. #BP399-1) diluted to 1x with deionized water to give 11.9 mM phosphate, 137mM NaCl, 2.7mM KCl, pH 7.4
- Tween 20 washing buffer, 0.05% in PBS 1X (PBS-T) – From 10X solution, dilute 1:10 in DI water. Alfa Aesar J63596
- 2-mercaptoethanol (BME) – Bio-Rad Cat# 1610710
- 2X Laemmli Sample Buffer; Bio-Rad Cat. #1610737
- 2X Reducing Sample Buffer – Laemmli with 5% BME
- Trans-Blot Turbo Transfer Packs – Midi 0.2 µm PVDF – Bio-Rad Cat# 1704157
- Trans-Blot Turbo Transfer Packs – Midi 0.2 µm Nitrocellulose – Bio-Rad Cat# 1704159
- Blotting-Grade Blocker – Bio-Rad Cat# 1706404
- Blocking buffer – 5% Blocker in 1X PBS-T
- Powder-free gloves

6. REAGENTS

- Sample Antigen to be tested
- Antibodies corresponding to sample antigens to be tested
- Detection Antibody – For Mouse: Goat anti-mouse antibody conjugated to horseradish peroxidase (GAM-HRP); Jackson Laboratories Cat. # 115-035-003 or equivalent. For Rabbit: Goat anti-rabbit antibody conjugated to horseradish peroxidase (GAR-HRP); Jackson Laboratories Cat. # 111-035-144 or equivalent.

7. PROCEDURE

7.1. Prepare Sample Antigen for PAGE.

7.1.1. For Recombinant protein: Prepare Intermediate Stock of sample antigen (Sample IS) by diluting antigen with PBS to a concentration of 0.1 mg/mL. Typical dilution scheme if starting antigen concentration is 1 mg/mL, add 10µL of antigen to 90µL of PBS (Sample IS). Then dilute (1:5): 1 part Sample IS with 4 parts Laemmli sample buffer to yield a final concentration of 0.02 mg/mL. Typical dilution scheme: add 100µL of Sample IS to 400µL of 2X Reducing buffer.

7.1.2. For Cell Lysate: Prepare an intermediate stock of cell lysate by diluting with 1X PBS to a concentration of 2 mg/mL. Dilute the intermediate stock 1:1 with 2X Reducing buffer.

7.1.3. Heat Sample Antigens at 95ºC for 5-10 minutes in a pre-heated water bath or heating block.

7.2. Gel Electrophoresis

7.2.1. Run approximately 0.2 ug of recombinant protein or approximately 20 ug of cell lysate (7.1.1, 7.1.2) on one-dimensional SDS-PAGE using a pre-cast, 4–20% Criterion™ TGX Stain-Free™ Protein Gel

7.3. Transfer onto membrane

7.3.1. Remove the gel from the electrophoresis rig and rinse the excess running buffer off of the plastic casing using DI water.

7.3.2. Crack open the gel casing carefully and use a razor blade to cut and remove the lower portion of the gel below the dye front.

7.3.3. Prepare the Transblot cassette with blotting sponges and membrane (nitrocellulose or PVDF) according to manufacturer's instructions.

7.3.4. Place the gel on top of the blotting membrane and make sure that is pressed flat against it with no air bubbles present.

7.3.5. Place the remaining blotting sponges on top of the gel and roll with a gel roller to compact and remove any remaining air bubbles; assemble the Transblot cassette and slot it into the machine.

7.3.6. Navigate to "Select Protocol->List->Bio-Rad->2 Mini or 1 Midi Gel->High MW" select run and then begin the procedure on the slot that contains the cassette.

7.4. Antibody Incubations

7.4.1. Place (nitrocellulose or PVDF) membrane in container containing ~50 mL of DI water to remove excess blotting buffer. Incubate for 5-10 minutes at room temperature with gentle rocking.

7.4.2. Remove DI water and add in ~50 mL of blocking buffer. Incubate for 1 hour at room temperature with gentle rocking.

7.4.3. Remove membrane and if needed cut the membrane into sections containing the wells needed for each antibody.

7.4.4. Place each membrane piece into separate mini western blot staining trays designated for each antibody.

7.4.5. Rinse each membrane in corresponding trays two times with ~25 mL of PBST.

7.4.6. Dilute antibodies to be evaluated to 0.1 ug/mL in 50 mL PBS-T.

7.4.7. Add diluted antibodies to mini trays containing the membranes.

7.4.8. Incubate with gentle rocking at 4°C overnight or for 1 hour at room temperature.

7.4.9. Rinse each blot two times with ~25 mL of PBS-T and gentle rocking for 5 minutes.

7.4.10. Dilute HRP-labeled detection antibody by adding 5 µL of appropriate secondary antibody to 50mL of PBS-T.

7.4.11. Add diluted HRP-labeled detection antibody to each mini tray containing the blots.

7.4.12. Incubate with gentle rocking for 1 hour at room temperature.

7.4.13. Rinse each blot two times with ~25 mL of PBS-T.

7.4.14. Prepare Opti-4CN and ECL substrate per kit instructions.

7.4.15. Add ~1-2 mL of ECL substrate (or as needed to cover the membrane) and incubate for up to 5 minutes (minimum 30 seconds).

7.4.16. Stop reaction by rinsing with DI water.

7.4.17. Image membrane using ChemiDoc (or equivalent) using Chemiluminescent Blot (647SP, No Light), Exposure: Auto Rapid 4x4.

7.4.18. After imaging, add ~1-2 mL Opti-4CN substrate to each membrane (or as needed to cover the membrane) and incubate up to 5 minutes as needed to visualize bands.

7.4.19. Stop reaction by rinsing with deionized water.

Scan membrane using ChemiDoc (or equivalent) using application: Colorimetric Blot (590/110, White Epi), Exposure: Auto Rapid 4x4

8. REFERENCED DOCUMENTS

8.1.1. http://www.jove.com/index/details.stp?ID=759

8.1.2. Bio-Rad ChemiDoc Imager Use Guide - https://www.bio-rad.com/webroot/web/pdf/lsr/literature/10000062126.pdf

8.1.3. Bio-Rad Trans-Blot Turbo Instruction Manual - https://www.bio-rad.com/webroot/web/pdf/lsr/literature/10000071567.pdf

8.1.4. Opti-4CN Substrate Kit Instruction Manual - https://www.bio-rad.com/webroot/web/pdf/lsr/literature/4100130.pdf

8.1.5. Bio-Rad Clarity and Clarity Max Western ECL Substrates Instruction Manual - https://www.bio-rad.com/webroot/web/pdf/lsr/literature/D085075.pdf