STANDARD OPERATING PROCEDURE

Title: Evaluation using Western Blot

SOP#: M-103

Version #: 1 **Author: R. Saul**

Date Approved: Feb. 5, 2009 **Date Modified:**

1. PURPOSE

The purpose of this document is to describe the procedure for performing Western Blot analysis for the characterization of proteins.

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

- Criterion™ Blotter assembly includes: Cell assembled with plate electrodes, Criterion gel holder cassette, fiber pad pack, gel blot assembly tray, sealed ice cooling unit, roller, magnetic stir bar; BioRad Cat. #170-4070
- Criterion™ Blotting Sandwiches includes: 0.2µm nitrocellulose transfer membrane and filter paper; BioRad Cat. #162-0232
- Labcor™ Mini Western Blot staining trays, 35x86x12.7mm; Continental Lab Products Cat. #731-002 or equivalent
- Magnetic stir plate
- BioRad PowerPac™ HC power supply or equivalent
- Rocking platform; LabLine model 4831 or equivalent
- Water bath or heating block with appropriate insert pre-heated to 90°C

- Epson photo quality scanner or equivalent
- Precision pipettes
- Additional equipment for one-dimensional gel electrophoresis (see SOP for SDS-PAGE, M-100)

5. MATERIALS

- Pre-cast, 18-lane 4-15% Tris-HCl polyacrylamide gel; BioRad Cat. #345-0023
- One dimension SDS-PAGE running buffer: 10x Tris/Glycine/SDS Buffer diluted to 1x with deionized water; BioRad Cat. #161-0732
- Protein molecular weight standards: Dual Color Precision Plus Protein™ Standards; BioRad Cat. #161-0374
- Coomassie Brilliant Blue 250 staining solution; BioRad Cat. #161-0436
- Transfer Buffer (Tris/Glycine with 20% Methanol): For each liter, add 100mL of 10x Tris/Glycine Buffer (BioRad Cat. #161-0734) to 700mL deionized water and 200mL of MeOH and pre-chill to 4ºC. Prepare at least 1.5 liters.
- TMB substrate (1-Step TMB-Blotting); Pierce-Thermo Scientific Cat. #34018
- 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
- PBS containing 0.1% Tween 20 (PBST): add 1mL of Tween 20 to 1L of PBS
- Laemmli Sample Buffer; BioRad Cat. #161-0737
- SuperBlock™ Blocking Buffer-Blotting in PBS; Pierce-Thermo Scientific Cat. #37517
- Powder-free gloves

6. REAGENTS

- Sample Antigen to be tested
- Antibodies corresponding to sample antigens to be tested
- Detection Antibody - Goat anti-mouse antibody conjugated to horseradish peroxidase (GAM-HRP); Jackson Laboratories Cat. # 115-035-003 or equivalent

- Mouse IgG, (10.0 mg/mL); Lampire Biological Laboratory Cat. #7404304 or equivalent

7. PROCEDURE

7.1. Prepare Sample Antigen for PAGE.

7.1.1. For Coomassie staining: Dilute Antigen to 1mg/mL in PBS. Prepare Antigen for PAGE run by diluting the 1mg/mL prep 1:4 with Laemmli Sample Buffer. Typical dilution scheme: 10µL of Antigen (1 mg/ml) diluted with 30µL of Laemmli Sample Buffer, yielding a final concentration of 0.25mg/mL.

7.1.2. For Western blot: 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): one 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 Laemmli Sample Buffer

7.1.3. Heat Sample Antigens at 90ºC for 5 minutes in a pre-heated water bath or heating block.

7.2. Prepare mouse IgG control

7.2.1. Prepare Control Intermediate Stock (Control IS) by serially diluting mouse IgG (10mg/mL) with PBS to a final concentration of 0.01 mg/mL. Typical dilution scheme: first dilute 10µL of mouse IgG with 90µL of PBS (1:10 dilution = 1 mg/ml), then dilute 10µL of the 1:10 solution with 990µL of PBS (1:100 dilution) yielding a final total dilution of 1:1000 (0.01 mg/ml).

7.2.2. Dilute (1:5) one part Control IS (0.01 mg/ml) with 4 parts Laemmli sample buffer to yield a final concentration of 0.002 mg/mL. Typical dilution scheme: add 100µL of Control IS to 400µL of Laemmli sample buffer

7.2.3. Heat at 90ºC for 5 minutes in a pre-heated water bath or heating block.

7.2.4. Note: unused Control IgG can be stored at -20ºC

7.3. Run prepared sample antigen (7.2.1) and control IgG (7.2.2) on one-dimensional SDS-PAGE following the procedure described in SOP M-100.

7.3.1. A typical SDS-PAGE run for a Western Blot of 3 antibodies using an 18-lane pre-cast gel is diagrammed below:

Layout of PAGE for Typical Western Blot

Lane	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18
Sample	mw	Ag		mw	Ag	IgG	mw		mw	Ag	IgG	mw		mw	Ag	IgG	mw	
Amt.	Std	2.5µg		Std	50ng	5ng	Std		Std	50ng	5ng	Std		Std	50ng	5ng	Std	
Vol.	10µL	10µL		10µL	2.5µL	2.5µL	5µL		10µL	2.5µL	2.5µL	5µL		10µL	2.5µL	2.5µL	5µL	
Use	Coomassie Staining			Antibody 1 blot					Antibody 2 blot					Antibody 3 blot				

7.3.2. Load a typical gel with samples and standards as follows:

7.3.2.1. 10µL of molecular weight standards in lanes 1, 4, 9, & 14

7.3.2.2. 5µL of molecular weight standards in lanes 7, 12 & 17

7.3.2.3. 10µL of the 0.25mg/mL antigen preparation in Lane 2

7.3.2.4. 2.5µL of the 0.02mg/mL antigen preparation in lanes 5, 10 & 15

7.3.2.5. 2.5µL of the 0.002 mg/ml Control IgG preparation in lanes 6, 11 & 16

7.4. Following completion of the SDS-PAGE run, open cassette housing and slice gel between Lanes 3 & 4 with razor blade. Place lanes 1-3 in shallow container and add enough Coomassie Blue R250 stain to cover the gel. Process gel following the staining procedure described in SOP M-100.

7.5. Remove the remaining gel from cassette housing (lanes 4-18) and place in 250mL 1x Transfer Buffer (pre-chilled to 4ºC) for 15 minutes with gentle rocking. Always wear gloves when handling membranes, filter paper, or gels to prevent contamination.

7.6. Set up transfer apparatus:

7.6.1. Fill the Criterion Blotter tank with 1x Transfer Buffer to about 50% of the fill volume.

7.6.2. Place a magnetic stir bar inside the tank

7.6.3. Place the ice block in the ice block pocket in the back of the cell. Flip down the lever to hold the ice block in place.

7.7. Gel & membrane sandwich set up:

7.7.1. Pour some chilled 1x Transfer Buffer into each compartment of the gel/blot assembly tray.

7.7.2. Place the cassette in the back/large compartment of the tray: Open the cassette so that the red side with handle is vertical (anode) and the black side (cathode) is lying horizontally and submerged in 1x Transfer Buffer.

7.7.3. Place a fiber pad on top of the black side of cassette, submerged in buffer. Push on the fiber pad with gloved finger tips to thoroughly wet the pad.

7.7.4. Place a piece of filter paper on top of the fiber pad (it should wet immediately).

7.7.5. Gently place the pre-equilibrated gel on top of the filter paper. Use roller to remove air bubbles that may be trapped under gel.

7.7.6. Slowly wet the nitrocellulose membrane in the 1x Transfer Buffer in front compartment by gradually submerging the membrane starting at one end and continuing until entire membrane is wet.

7.7.7. Remove wet membrane from the front compartment and place it on top of the gel, taking care not to trap air. The membrane should not be moved or adjusted once it touches the gel. Use roller to roll out bubbles.

7.7.8. Place a piece of filter paper on top of the membrane. Run the roller gently over the top of the filter paper to remove any air bubbles trapped in the sandwich.

7.7.9. Wet a second fiber pad in the front compartment of the tray, again using finger tips to completely saturate the pad. Then place the wet fiber pad on top of the second filter paper.

7.7.10. Lower the clamp-side of the cassette and lock In the closed position.

7.8. Blot Transfer

7.8.1. Move the locked cassette into the groove in the blotter tank, aligning the red side of the card with the red electrode. Make sure the magnetic stirrer is free to move.

7.8.2. Add remaining 1x Transfer Buffer to the fill level marked on the tank and place tank on magnetic stir plate. Turn on stir plate to low setting.

7.8.3. Put lid on transfer tank, plug the cables into the power supply

7.8.4. Set power supply to 100V and press start. Run the blot for 30 minutes.

7.8.5. Upon completion of the run, disassemble the blotting sandwich and remove the membrane for development (6.9). Clean the cell, fiber pads and cassette with multiple rinses of deionized water.

7.9. Blot Development

7.9.1. Place membrane in container containing 100 mL of SuperBlock Blocking Buffer-Blotting. Incubate for 15 minutes with gentle rocking.

7.9.2. Remove membrane and slice into 3 segments between wells 8/9 and 13/14 as described in the diagram (6.3.1). The slicing can be done with a razor blade or scalpel.

- Note: the diagram is just an example and the order and location will depend on the number of antibodies to be evaluated and the number of wells used per evaluation. .

7.9.3. Place sliced blot segments into separate mini western blot staining trays designated for each antibody.

7.9.4. Rinse each blot in tray with 10 mL of PBST.

7.9.5. Dilute antibodies to be evaluated1:5000 in PBST: Add 2µL of 1mg/mL antibody solution to 10 mL of PBST.

7.9.6. Add diluted antibodies to mini trays containing the blots.

7.9.7. Incubate with gentle rocking for 30 minutes.

7.9.8. Rinse each blot 3x with 10mL of PBST.

7.9.9. Dilute HRP-labeled detection antibody 1:7500: add 4µL of goat anti-mouse HRP antibody to 30mL of PBST.

7.9.10. Add 10 mL of diluted HRP-labeled detection antibody to each mini tray containing the blots.

7.9.11. Incubate with gentle rocking for 30 minutes.

7.9.12. Rinse each blot 3x with 10 mL of PBST.

7.9.13. Transfer each blot to clean, unused mini trays and rinse with an additional 10 mL of PBST.

7.9.14. Add 3 mL of TMB substrate and incubate for approximately 5 to 10 minutes, or until the control IgG line has developed.

7.9.15. Stop reaction by rinsing with deionized water.

7.9.16. Remove membranes and allow to air dry.

7.9.17. Scan the final developed blots using a photo quality scanner such as an Epson Perfection 4490 Photo or equivalent.

8. REFERENCED DOCUMENTS

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

8.1.2. http://www.afcs.org/reports/v1/BC0001/Protocols/Protein%20Transfer%20BIORAD.pdf

8.1.3. Bio-Rad Criterion™ Blotter assembly Instruction Manuals

8.1.4. SOP M-100: SDS-PAGE Evaluation

8.1.5. SOP M-101: Protein Determination by BioRad Protein Assay