

STANDARD OPERATIONS PROCEDURES FOR THE COMMON FUND: PROTEIN CAPTURE REAGENTS PROGRAM (western Blot)

1. PURPOSE

The purpose of this document is to describe the procedure for performing a **typical** Western Blot analysis for the characterization of human fragment antigen-binding (Fab) and monoclonal antibodies.

2. SCOPE

This procedure may be used for proteins with molecular weights in the range of 2 KDa to 250 KDa. 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. MATERIALS

- Pre-cast, 18-well, 30 μ L, 4-20% Tris-HCl Criterion™ polyacrylamide gel (Bio-Rad, Cat. #345-0033)
- One dimension SDS-PAGE running buffer: 10x Tris/Glycine/SDS Buffer (Fisher Scientific, Cat. #BP1341-4) diluted to 1x with deionized water
- Protein molecular weight standards: Bio-Rad Dual Xtra Precision Plus Protein™ Standards (Bio-Rad, Cat. #161-0377)
- Bio-Rad PowerPac™ HC power supply
- Criterion™ Cell (Bio-Rad, Cat. #165-6001)
- Powder-free gloves
- Trans-Blot® Turbo™ Transfer System (Bio-Rad, Cat. #170-4155)
- Opti-4CN Substrate Kit (Bio-Rad, Cat. #170-8235)
- Trans-Blot® Turbo™ Midi Nitrocellulose Transfer Pack (Bio-Rad, Cat. #170-4159)
- Opti-4CN substrate (Bio-Rad, Cat. #170-8235)

- 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 and pH 7.4
- PBS containing 0.1% Tween-20 (PBST): add 1mL of Tween 20 (ACROS ORGANICS, Cat. #233362500) to 1L of PBS
- Laemmli Sample Buffer (Bio-Rad, Cat. #161-0737)
- Blotting-Grade Blocker (Bio-Rad, Cat. #170-6404): add 5g to 100mL PBS
- Ultra Cruz™ Gel Incubation Tray, 200 mL (Santa Cruz Biotechnology, Inc., Cat. #sc-201756)
- ChemiDoc™ XRS+ System with Image Lab™ Software, Bio-Rad, Cat. #170-8265
- Containers for pre-protein detection and blocking
- Reversible Protein Detection Kit (Sigma, Cat. #R-PROB)
- Rocking platform – LabLine model 4831 or equivalent
- Water bath or heating block with appropriate insert pre-heated to 90°C.

5. REAGENTS

5.1. For mouse monoclonal antibody evaluation

- Antigen corresponding to the antibody.
- Purified monoclonal antibody corresponding to the Antigen
- Goat anti-mouse - horseradish peroxidase (GAM-HRP) labeled antibody; Jackson ImmunoResearch Laboratories, Cat. #115-035-166

5.2. For human Fab binder evaluation

- Antigen corresponding to the antibody.
- Purified human Fab antibody corresponding to the Antigen
- Goat anti-human - horseradish peroxidase (GAH-HRP) labeled antibody; Jackson ImmunoResearch Laboratories, Cat. #109-036-097

6. PROCEDURE

6.1. Prepare Sample Antigen for PAGE

- 6.1.1. 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 5 µL of antigen to 45 µL of PBS (Sample IS).

6.1.2. Dilute one part Sample IS with three parts Laemmli sample buffer to yield a final concentration of 0.025 mg/mL. Typical dilution scheme: add 10 µL of Sample IS to 30 µL of Laemmli sample buffer.

6.1.3. Heat Sample Antigens at 90°C for 5 minutes in a water bath or heating block.

6.2. Prepare Mouse IgG (1°Antibody) for Control (antibody to be evaluated)

6.2.1. Prepare Control Intermediate Stock (Control IS) by diluting 1° Antibody with PBS to a concentration of 0.005 mg/mL. Typical dilution scheme: if starting antibody concentration is 1 mg/mL, add 2 µL of 1° Antibody with 398 µL of PBS.

6.2.2. Dilute one part Control IS with three parts Laemmli sample buffer to yield a final concentration of 0.00125 mg/mL. Typical dilution scheme: add 10 µL of Control IS to 30 µL of Laemmli sample buffer.

6.2.3. Heat at 90°C for 5 minutes in a water bath or heating block.

6.3. Run molecular weight standards, prepared sample antigen (6. 1) and 1° Antibody control (6. 2) on one-dimensional SDS-PAGE according to the Criterion™ Cell Instruction Manual.

6.3.1. A typical SDS-PAGE run for a Western Blot of 6 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	1° Ab	Mw	Ag	1° Ab	Mw	Ag	1° Ab	Mw	Ag	1° Ab	Mw	Ag	1° Ab	Mw	Ag	1° Ab
	Std			Std			Std			Std			Std			Std		
Amt.		500ng	25ng		500ng	25ng		500ng	25ng		500ng	25ng		500ng	25ng		500ng	25ng
Vol.	10µL	20µL	20µL	10µL	20µL	20µL	10µL	20µL	20µL	10µL	20µL	20µL	10µL	20µL	20µL	10µL	20µL	20µL
Use	Antibody 1 Blot			Antibody 2 Blot			Antibody 3 Blot			Antibody 4 Blot			Antibody 5 Blot			Antibody 6 Blot		

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

6.3.2.1. 10 µL of molecular weight standards in lanes 1, 4, 7,10 13,&16

6.3.2.2. 20µL of the 0.025 mg/mL antigen preparation in lanes 2, 5, 8, 11, 14 & 17

6.3.2.3. 20µL of the 0.00125 mg/ml 1° Antibody Control preparation in lanes 3, 6, 9, 12, 15 & 18

6.3.2.4. Run gel at 150V for 1 hr. and 10 min. Run until dye front is about 0.5 in. from bottom of gel.

6.4. Following completion of SDS-PAGE run, carefully open cassette housing.

6.4.1. Use razor blade to trim off gel wells and cut below dye front to remove excess gel.

Note: Always wear gloves when handling gels, membranes and filter paper to prevent contamination.

6.5. Transfer Apparatus Set Up:

6.5.1. Rinse blotting cassette electrodes (top (-) and bottom (+)) with deionized water and dry with paper towels.

6.6. Gel & Membrane Sandwich Set Up:

6.6.1. Upon opening the Midi trans-blot package you will see the membrane & left and right finger tabs labeled Top (-) and Bottom (+), respectively. Using the right finger tab, remove bottom ion reservoir stack with membrane and place on top of bottom (+) cassette electrode while exposing membrane on top.

6.6.2. Gently remove gel from cassette housing (lanes 1-18) and carefully center gel on top of membrane. Use blot roller to remove air bubbles that may be trapped under gel and membrane.

6.6.3. Using the left finger tab, remove top ion reservoir stack and place on top of gel. Roll the blot roller gently over the top of reservoir to remove any air bubbles trapped in the blotting sandwich.

6.6.4. Lower top (-) cassette electrode on top of the blotting sandwich and lock dial in the closed position.

6.7. Blot Transfer

6.7.1. Insert trans-blot cassette into cassette chamber of Trans-Blot[®] Turbo[™] instrument. Use "High MW 2.5A – 25V, 10M" protocol and start blot transfer according to Trans-Blot[®] Turbo[™] Instrument Manual.

6.7.2. Following completion of blot transfer, disassemble the blotting sandwich and remove the membrane for pre-protein detection and development.

6.7.3. Rinse blotting cassette electrodes (top (-) and bottom (+)) with deionized water and dry with paper towels or air dry overnight.

6.8. Pre-protein Blot Detection and Development

6.8.1. The pre-protein detection step is done to ensure protein is present before developing the membrane.

- 6.8.2. Clean two containers for pre-protein detection by rinsing with methanol and wiping dry with paper towels.
- 6.8.3. Place membrane in a clean container and add deionized water to cover membrane. Gently swirl periodically for 5 min. Discard deionized water and repeat rinse 1x.
- 6.8.4. Prepare reversible protein detection mix by adding 10 mL of Buffer A and 10 mL of Buffer B into a 50 mL conical tube. Mix well.
- 6.8.5. Add 5 mL of A & B mix on top of membrane and gently shake to view protein bands (about 1- 2 min.). More A& B mix can be added to membrane to enhance visibility of protein bands but be mindful of increasing background.
- 6.8.6. Remove membrane from container and place on ChemiDoc XRS+ Molecular Imager or equivalent platform.
- 6.8.7. Scan and save membrane image according to ChemiDoc XRS+ Molecular Imager or equivalent Manual.
- 6.8.8. Place membrane back into container with detection mix. Pour off detection mix. Add PBS to cover membrane and gently swirl to remove detection/stain. Repeat 2-3x.

6.9. Blot Development

- 6.9.1. Prepare Blotting-Grade Blocker by adding 5 g in 100 mL PBS.
- 6.9.2. Place membrane into a clean container and add 50 mL of Blotting-Grade Blocker.
- 6.9.3. Incubate at room temp for 15 – 30 min.
- 6.9.4. Remove membrane and slice into 6 segments between wells 3/4, 6/7, 9/10, 12/13 and 15/16 as described in the diagram (6.3.1). The slicing can be done with a razor blade or scalpel.
Note: This depends on the number of antibodies to be evaluated and the number of wells used per evaluation. The diagram is just an example.
- 6.9.5. Place sliced membrane segments into separate small incubation trays designated for each antibody and add 20 mL of Blotting-Grade Blocker.
- 6.9.6. Dilute antibodies to be evaluated 1:5000 in Blotting-Grade Blocker by adding 4 μ L of 1 mg/mL antibody solution to small incubation tray with 20 mL of Blotting-Grade Blocker.
- 6.9.7. Incubate at room temp with gentle rocking for 60 minutes.
- 6.9.8. Rinse each membrane 2x with 20 mL of PBST.

- 6.9.9. Add 20 mL of PBST to each incubation tray with membrane segments.
- 6.9.10. Dilute HRP-labeled detection antibody 1:4000 in PBST by adding 5 μ L of 1 mg/mL GAM-HRP or GAH-HRP antibody solution to small incubation trays with 20 mL of PBST.
- 6.9.11. Incubate at room temp with gentle rocking for 60 minutes.
- 6.9.12. Rinse each membrane 2x with 20 mL of PBST.
- 6.9.13. Transfer each membrane to clean, unused small incubation tray and rinse with an additional 20 mL of PBST.
- 6.9.14. Prepare Opti-4CN Substrate mix by adding 1 mL Diluent to 9 mL deionized water and 0.2 mL Substrate. Mix well.
- 6.9.15. Add 3 mL of Opti-4CN Substrate mix to each small incubation tray with membrane and incubate for approximately 5 to 10 minutes, or until the 1^o Antibody Control protein band has developed.
- 6.9.16. Stop reaction by adding 20 mL of deionized water to small incubation tray with membrane.
- 6.9.17. Remove membranes and allow to air dry or place between two paper towels to absorb excess liquid.
- 6.9.18. Put membranes in correct order to mimic the original sample order of whole membrane on Bio-Rad ChemiDoc XRS+ Molecular Imager or equivalent platform.
- 6.9.19. Scan and save the final developed membranes according to Bio-Rad ChemiDoc XRS+ Molecular Imager or equivalent Manual.

7. REFERENCED DOCUMENTS

- 7.1. Instructions for use, CriterionTM Cell
- 7.2. Instructions for use, Trans-Blot[®] TurboTM Midi Nitrocellulose Transfer Pack
- 7.3. Operation Manual, Trans-Blot[®] TurboTM Instrument
- 7.4. Operation Manual, ChemiDocTM XRS+ System with Image LabTM Software