STANDARD OPERATING PROCEDURE

Title: Antibody Evaluation by Immunoblot (Western Blot) Analysis

SOP#: M-103

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1. PURPOSE

The purpose of this procedure is to evaluate the performance of monoclonal antibodies using traditional immunoblot (Western blot) technique.

2. SCOPE

This procedure may be used to detect proteins or conjugated peptides with molecular weights that range from 10 KDa to 250 KDa. Other procedures must be used to detect proteins or peptides with molecular weights that lie outside of this range. The described multiplex assay can be used for the detection of recombinant protein (purified or in over-expressed lysate) or for the detection of the endogenous protein in whole cell lysates or tissue homogenates. If the recombinant protein is tagged, the protein should be tested with anti-tag antibody simultaneously to verify the expected molecular weight (Myc tag is the most common). It is required to include a housekeeping protein(s) that can be used as a loading control in assays where whole cell lysates or tissue homogenates are probed. This procedure describes how to detect the target protein and housekeeping protein simultaneously by using primary antibodies against Cytochrome C or Vinculin of a different host compared to the test antibody.

3. RESPONSIBILITIES

It is the responsibility of person (s) performing this procedure to be familiar with laboratory safety procedures, and to always wear appropriate Personal Protective Equipment (PPE). The interpretation of results must be done by a person trained in the procedure and familiar with such interpretation.

4. EQUIPMENT

4.1. Trans-Blot Turbo Transfer System – BioRad Cat# 1704150

4.2. Labcor™ Mini Western Blot staining trays, 35 x 86 x 12.7 mm; Continental Lab Products Cat. #731-002 or equivalent

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4.3. Magnetic stir plate

4.4. Rocking platform; LabLine model 4831 or equivalent

4.5. Water bath or heating block with appropriate insert pre-heated to 95°C

4.6. ChemiDoc MP Imaging System (BioRad Cat # 12003154) or equivalent

4.7. Precision pipettes

4.8. PowerPac™ Basic Power Supply Biorad 1645050

4.9. Criterion™ Cell Biorad #1656001

4.10. Gel Roller Biorad #1651279

5. MATERIALS

5.1. Pre-cast, 4–20% Criterion™ TGX Stain-Free™ Protein Gel, 18 well, 30 µl BioRad Cat. #5678094

5.2. One-dimension SDS-PAGE running buffer: 10x Tris/Glycine/SDS Buffer; BioRad Cat. #1610732

5.3. Protein molecular weight standards: Precision Plus Protein™ All Blue Standards; BioRad Cat. #1610373

5.4. Clarity Western ECL Substrate – BioRad Cat. #1705061

5.5. Phosphate Buffered Saline (PBS), 10x solution (Fisher Scientific Cat. #BP399-1)

5.6. 10x Tris Buffered Saline (TBS) – BioRad Cat# 1706435

5.7. TBS Tween buffer 20X (Thermo Fisher, cat #28360)

5.8. 2-mercaptoethanol (BME) – BioRad Cat# 1610710

5.9. 2X Laemmli Sample Buffer; BioRad Cat. #1610737

5.10. 1X RIPA Buffer, Thermo Fisher Cat. # 89901

5.11. Trans-Blot Turbo Transfer Packs – Midi 0.2 µm Nitrocellulose – BioRad Cat# 1704159

5.12. Blotting-Grade Blocker – BioRad Cat# 1706404

5.13. Blocking Buffer – 5% goat serum in 1X TBS

5.14. Powder-free gloves

6. REAGENTS

6.1. Sample lysate (i.e. whole cell, tissue homogenate, or over-expressed lysate), sample purified protein (tagged or untagged), or sample

conjugated peptide (i.e. BSA-conjugated) that is known to contain the target protein or peptide. When possible, include a negative control sample that does not express the target protein or peptide in the procedure.

6.2. Monoclonal antibodies (purified) to be tested

6.3. Mouse Anti-Cytochrome C antibody (Abcam, Cat. # ab110325)

6.4. Rabbit Anti-Cytochrome C antibody (Abcam, Cat. # ab133504)

6.5. Mouse Anti-Vinculin (Abcam, Cat. # ab130007)

6.6. Rabbit Anti-Vinculin (Abcam, Cat. # ab 129002)

6.7. Mouse Anti-myc (Cell Signaling, Cat. # 2276S

6.8. Rabbit Anti-myc (Cell Signaling, Cat. # 2278S

6.9. Goat serum (EMD Millipore. Cat. # S26-LITER, EMD Millipore)

6.10. *ECL detection secondary antibodies for target protein detection*:

6.10.1. For mouse primary antibodies against target protein: Goat anti-mouse antibody conjugated to horseradish peroxidase (GAM-HRP); Jackson Laboratories Cat. # 115-035-003 or equivalent.

6.10.2. For rabbit primary antibodies against target protein: Goat anti-rabbit antibody conjugated to horseradish peroxidase (GAR-HRP); Jackson Laboratories Cat. # 111-035-144 or equivalent.

6.11. *Fluorescently labeled secondary antibodies for target protein detection*:

6.11.1. For mouse primary antibodies against target protein: Alexa-Fluor 790 conjugated to goat anti-mouse (Jackson Labs, Cat# 115-655-146)

6.11.2. For rabbit primary antibodies against target protein: Alexa-Fluor 790 conjugated to goat-anti rabbit (Jackson Labs, Cat# 111-655-144)

6.12. *Fluorescently labeled secondary antibodies for housekeeping protein or tag detection*:

6.12.1. For mouse primary antibodies against housekeeping protein or tag: Alexa-Fluor 488 conjugated to goat anti mouse secondary antibody (Jackson Labs, Cat# 111-545-146)

6.12.2. For Rabbit primary antibodies against housekeeping protein or Myc tag: Alexa-Fluor 488 conjugated to goat anti rabbit secondary antibody (Jackson Labs, Cat# 111-545-144)

7. PROCEDURE

7.1. Prepare Sample(s) for SDS-PAGE

7.1.1. **Preparation of reducing buffer**: Mix 950 uL of 2X Laemmli buffer and 50 uL of BME.

7.1.2. **Preparation of PBS buffer**: Dilute 10X PBS buffer to 1X in diH2O.

7.1.3. **For recombinant protein(s) and BSA-conjugated peptides:** Prepare Intermediate Stock of sample protein (Sample IS) by diluting protein with 1X PBS to a concentration of 0.1 mg/mL. For example, if starting protein concentration is 1 mg/mL, add 10µL of protein to 90µL of PBS (Sample IS). Then dilute 1:2, 1 part Sample IS with one part reducing sample buffer to yield a final concentration of 0.05 mg/mL. Typical dilution scheme: add 100µL of Sample IS to 100µL of 2X Reducing buffer.

7.1.4. **For cell lysate/over-expressed cell lysate:** Prepare an Intermediate Stock of cell lysate (Sample IS) by diluting with 1X RIPA lysis buffer to a concentration of 2 mg/mL. Then dilute 1:2, 1 part Sample IS with 1 part 2X Reducing buffer, to yield a final concentration of 1 mg/mL. Typical dilution scheme: add 100µL of Sample IS to 100µL of 2X Reducing buffer.

7.1.5. Heat Samples at 95ºC for 5-10 minutes in a pre-heated water bath or heating block.

7.2. Gel Electrophoresis

7.2.1. Dilute SDS-PAGE Running Buffer to 1X with deionized (DI) water.

7.2.2. Assemble gel electrophoresis rig with the pre-cast gel.

7.2.3. Fill the chamber with running buffer up to the fill line.

7.2.4. Load an appropriate quantity of sample on a one-dimensional SDS-PAGE, pre-cast, 4–20% Criterion™ TGX Stain-Free™ Protein Gel:

- Recombinant protein: 0.2 µg – 0.5 µg
- Over-expressed lysate: 10 µg – 20 µg
- Whole cell lysate: 20 – 40 µg
- Tissue homogenate lysate: 20 – 40 µg
- BSA-conjugated peptide: 0.2 µg – 0.5 µg

7.2.5. Close the chamber with the lid and connect the lid to the power supply.

7.2.6. Run electrophoresis at 50 volts for 5 minutes, then 250 volts for 25 minutes.

7.3. **Transfer onto nitrocellulose membrane**

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

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

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

7.3.4. Place the gel on top of the blotting membrane and make sure that it is pressed flat against it with no air bubbles present. If there are bubbles, gently press them out.

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 transfer 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. Preparation of TBS-T buffer (0.05% Tween in TBS 1X) – From 20X solution, dilute 1:20 in DI water.

7.4.2. The primary and secondary antibody incubation conditions are project specific and should be carefully evaluated. These conditions should be evaluated individually for each antibody. This evaluation is based upon various factors which include:

7.4.2.1. <u>Target primary antibody host</u>: the host of the primary antibody against the target protein always needs to be different from the host of the primary antibody against the housekeeping or the tag. For example, if the host of the antibody against the target protein is mouse, the host for the antibody against the housekeeping needs to be rabbit.

7.4.2.2. <u>Concentration of primary antibody against the target</u>: if the primary antibody has a concentration ≥ 1 mg/mL, proceed with fluorescence detection. If the concentration < 1 mg/mL, proceed with HRP tagged secondary antibody detection (ECL detection). Housekeeping and tag detection will always be fluorescence.

7.4.2.3. Substrate type: when using HRP secondary antibodies, it is best to separate over-expressed lysate/recombinant protein samples from whole cell lysates to avoid detection issues (strong signal from recombinant protein in solution or over-expressed lysates might mask the lower intensity signal from cell lysates).

Below Table 1 serves as a guide to make such an evaluation.

Table 1

Ab target	*Primary Ab Concentration*	*Primary Ab host*	*Primary Ab dilution*	*Secondary Ab*	*Secondary Ab Dilution*
Target	≥ 1 mg/mL	Mouse	1:500	Goat anti-Mouse AF790	1:50,000
Target	≥ 1 mg/mL	Rabbit	1:500	Goat anti-Rabbit AF790	1:50,000
*Housekeeping or Tag**	≥ 1 mg/mL	Mouse	1:10,000	Goat anti-Mouse AF488	1:50,000
*Housekeeping or Tag**	≥ 1 mg/mL	Rabbit	1:10,000	Goat anti-Rabbit AF488	1:50,000
Target	< 1 mg/mL	Mouse	1:10,000	Goat anti-Mouse HRP	1:10,000
Target	< 1 mg/mL	Rabbit	1:10,000	Goat anti-Rabbit HRP	1:10,000

Note: Dilutions from stock 1 mg/mL

**: Commercially available antibodies against housekeeping protein or tag are generally ≥ 1 mg/mL*

7.4.3. Place (nitrocellulose) 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.

7.4.4. Prepare fresh blocking buffer, mixing 95 mL of TBS-T and 5 mL of goat serum.

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

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

7.4.7. Place each membrane piece into separate and labeled mini western blot staining trays designated for each antibody.

7.4.8. Rinse each membrane in corresponding trays two times with ~25 mL of TBS-T.

7.4.9. Select and dilute primary antibodies according to Table 1 in TBS-T (section 7.4.2).

7.4.10. Add diluted antibodies to mini trays containing the corresponding membranes.

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

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

7.4.13. Dilute secondary detection antibody in TBS-T according to the guidelines shown in Table 1.

7.4.14. Add the diluted secondary detection antibody to each mini tray containing the corresponding membranes.

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

7.4.16. Rinse each membrane two times with ~25 mL of TBS-T.

7.4.17. Proceed to detection procedure using ChemiDoc (or equivalent). If detection of the protein target is fluorescence based, refer to section 7.4.17.1; if detection of the target protein is ECL based, refer to section 7.4.17.2

7.4.17.1. Fluorescence based target protein detection: Image membrane using ChemiDoc (or equivalent) using the following settings:

7.4.17.1.1. Colorimetric Blot (590/110, White Epi) Exposure: Auto Rapid, for protein ladder

7.4.17.1.2. Alexa 790 Blot (835/50, IR Epi), Exposure: Auto Rapid 3x3, for target

7.4.17.1.3. Alexa 488 Blot (532/28, Blue Epi) Exposure: Auto Rapid. For housekeeping or tag

7.4.17.2. ECL based protein target detection.

7.4.17.2.1. Prepare ECL substrate per kit instructions.

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

7.4.17.2.3. Stop reaction by rinsing with DI water.

7.4.17.2.4. Image membrane using ChemiDoc (or equivalent) using the following settings:

7.4.17.2.4.1. Colorimetric Blot (590/110, White Epi) Exposure: Auto Rapid, for protein ladder

7.4.17.2.4.2. Chemiluminescent Blot (647SP, No Light), Exposure: Auto Rapid 4x4, for target

7.4.17.2.4.3. Alexa 488 Blot (532/28, Blue Epi), Exposure: Auto Rapid, for housekeeping or tag

7.5. **Data Evaluation**

7.5.1. Review the blot image as follows:

7.5.1.1. If there is a single band and

7.5.1.1.1. It is migrating at a molecular weight that is +/- 20% of the expected molecular weight as reported on Uniprot (www.uniprot.org), the test result is positive.

7.5.1.1.2. It is migrating at a molecular weight that is > 20% of the expected molecular weight as reported on Uniprot (www.uniprot.org), and the target is known to be expressed with a post-translational modification such as glycosylation, the test result may be positive.

7.5.1.2. If there are multiple bands and the most intense band is migrating at a molecular weight that is +/- 20% of the expected molecular weight that is reported on Uniprot (www.uniprot.org), the test result is presumed positive.

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