Western Blot Protocols- Quality Control Protocol

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Protocols

R&D Systems Quality Control laboratories use these Western blotting and immunostaining protocols to show that our polyclonal and monoclonal antibodies are specific for the proteins they were raised against and to determine the sensitivity of the antibody for its antigen.

Protein samples are prepared with SDS and run under both reduced and non-reduced conditions on appropriate SDS-PAGE gel. The proteins are transferred to a PVDF membrane using a semi-dry transfer apparatus. For those proteins that have not been tested with natural samples, a protocol and troubleshooting guide is provided for Western blot optimization. Please note that for the antibodies that have been validated for natural samples, an optimized protocol is provided on the datasheet, which should be suitable for most samples.

Western Blot Materials	
Semi-dry Transfer Apparatus	Bio-Rad (Catalog # 170-3940) or equivalent
Power Supply	0 - 100 VDC (adj. current to 1 Amp)
PVDF Membrane	0.45 µm pore size; cut to same size as gel (Millipore, Catalog # IPVH304-FO) or equivalent
Filter Paper	Schleicher & Schuell 3MM or equivalent, cut to same size as gel
Wetting Solution	100% Methanol
Gel Running Buffer	193 mM Glycine, 2.5 mM Tris (Base), 0.1% SDS
Anode Buffer I	300 mM Tris, 20% methanol, pH 10.4
Anode Buffer II	25 mM Tris, 20% methanol, pH 10.4
Cathode Buffer	25 mM Tris, 20% methanol, 40 mM 6-aminocaproic acid, pH not adjusted
Sample Buffer	6% SDS, 0.25 M Tris, pH 6.8, 10% glycerol, 10 mM NaF and bromophenol blue with or without 20 mM dithiothreitol (DTT)
Additional Tools	Forceps, clean plastic test tube, gloves, razor blade

- Electrophoresis
- Transfer of Protein
- Immunostaining

Electrophoresis:

1. Selection of a SDS-PAGE gel. Typically 12% acrylamide gels are used for high molecular weight (MW) proteins (>50 kDa), 15% gels for mid range MW proteins (15 - 50 kDa), and 20% gels for low MW proteins (<15 kDa).
2. Treatment of samples: Add equal volumes of either 2X reducing or non-reducing Sample Buffer to protein sample solutions, vortex gently to mix, and heat in boiling water bath for 5 minutes.
3. Clamp the gel into an electrophoresis apparatus. Add Gel Running Buffer.
4. Load samples onto gel.
5. Connect electrophoresis unit to power supply.
6. Start the electrophoresis process for the samples at 20 mA per gel. Run gel at constant current.
7. Once the dye front has completely migrated into the running gel, increase current to 30 mA per gel.
8. When the dye front reaches to about ½ the length of the running gel, increase current to 40 mA per gel.
9. Electrophoresis is complete when the dye front migrates about 2 mm from the bottom of the gel.

Transfer of protein: (See Figure 1)

Note: Always wear gloves or use forceps when handling blotting membranes to avoid contamination with protein from fingers.

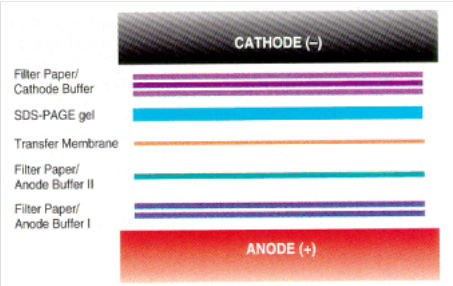

Fig. 1. Semi-dry transfer stack assembly.

1. **Preparation of PVDF membrane:**
    a. Wet membrane by laying it on the surface of methanol for 15 seconds. The membrane should change from opaque to semi-transparent.
    b. Carefully place membrane in de-ionized water and soak for 2 minutes.
    c. Carefully place the membrane in Anode Buffer II for 5 minutes.
2. **Assembly of transfer unit: (See Figure 1)**
    a. Wet two pieces of filter paper in Anode Buffer I and place on anode plate of apparatus. Avoid trapping air bubbles
    b. Wet one piece of filter paper in Anode Buffer II and place on top of filter paper stack.
    c. Remove PVDF membrane from Anode Buffer II and place on top of filter paper stack.
    d. Place gel on top of PVDF membrane taking care not to trap air bubbles between gel and membrane.
    e. Wet three pieces of filter paper in Cathode Buffer and place on top of gel. Use a clean plastic test tube to roll out air bubbles.
    f. Place cathode plate of blotter on top transfer stack.
3. **Transfer conditions and handling of membrane after transfer:**
    a. Connect high voltage cords to power supply. Apply a constant current of 1.9 - 2.5 mA per cm^2 of gel area for 30 - 60 minutes. Appropriate transfer time must be determined empirically.
    b. After transfer is complete, turn off power supply and remove cathode plate of blotter. Remove transfer membrane and cut lower right corner of membrane to mark orientation of the membrane.
    c. Discard the first two layers of filter paper and gel. Mark the bands of the molecular weight markers on the membrane with a ballpoint pen.
    d. Dry membrane in one of two ways. For fast drying, soak the membrane in 100% methanol for 10 seconds to drive out the water. Then, place the membrane on a piece of paper towel and leave on a lab bench for 15 minutes. For slower drying, place membrane on a piece of paper towel and leave on a lab bench for 2 hours or overnight.
    e. The dried membrane can be used immediately or stored in a dry container at 4°C for later use.

Immunostaining:

Bring all the solutions and reagents to room temperature before use; otherwise the detection limit may be compromised.

1. **Blocking:**
    a. If membrane was dried and stored at 4°C, re-wet in 100% methanol for a few seconds. Discard methanol and rinse membrane in de-ionized water.
    b. Block unoccupied protein binding sites on membrane by placing membrane in Blocking Buffer. Incubate on rocker/shaker at room temperature for 1-2 hours. Pour off the Blocking Buffer.
2. **Incubation with primary antibody:**
    a. Prepare primary antibody in Primary and Secondary Antibody Solution at the concentration listed in the table below for different types of antibodies. Add sufficient Primary Antibody Solution with enough volume to cover the entire blot.

Antibody Category	Working Concentration (microgram/mL)
Non Affinity Purified Polyclonal Antibody	1 - 2
Affinity Purified Polyclonal Antibody	0.1 – 0.2
Biotinylated Polyclonal Antibody	0.1 – 0.2
Monoclonal Antibody	1 – 2

    b. Place container on a rocker and incubate at room temperature for one hour. Alternatively, the primary antibody incubation can be carried out at 4°C overnight to improve detection limit.
    c. Pour off Primary Antibody Solution and rinse membrane twice with de-ionized water. Then wash membrane twice in Wash Solution for 15 minutes each with shaking.
3. **Incubation with biotinylated secondary antibody:**
    Note: if the primary antibody is biotinylated, this step should be omitted and proceed directly to next step.
    a. Prepare biotinylated species-specific antibody in Primary and Secondary Antibody Solution to pre-determined dilution. Add to blot container at volume sufficient to cover the entire membrane.
    b. Incubate on a rocker at room temperature for 30 minutes.
    c. Pour off biotinylated secondary antibody solution and rinse membrane twice with de-ionized water. Then wash membrane twice in Wash Solution for 20 minutes each with shaking.

sufficient to cover the entire membrane.

b. Incubate on a rocker at room temperature for 30 minutes.

c. Pour off SA-AP solution, rinse membrane twice with de-ionized water. Then wash membrane twice in Wash Solution for 20 minutes each with shaking.

5. **Color Development**

a. **Preparation of substrate solution:** Add 132 microliters of Color Reagent A stock solution to 20 microliters of AP Substrate Buffer and mix well. Add 66 microliters of Color Reagent B stock solution and mix well. Prepare substrate solution just before color development and use within 1 hour.

b. **Color development:** To a washed membrane in a suitable container, add 20 mL of substrate solution and develop at room temperature with gentle shaking for 15 minutes or until desirable bands are developed.

c. **Stopping of color development:** Pour off the substrate solution and rinse the membrane with de-ionized water three times.

Immunostaining Materials	
Primary (1°) Antibody	Any polyclonal or monoclonal antibodies that have been validated for use in Western blots
Secondary (2°) Antibody	Depends on 1° antibody utilized, for example: • Biotinylated donkey anti-goat IgG R&D Systems Catalog # BAF109 or equivalent • Biotinylated mouse anti-hamster IgG R&D Systems Catalog # BAM011 or equivalent • Biotinylated goat anti-mouse IgG R&D Systems Catalog # BAF007 or equivalent • Biotinylated goat anti-rabbit IgG R&D Systems Catalog # BAF008 or equivalent • Biotinylated goat anti-chicken IgY R&D Systems Catalog # BAF010 or equivalent *Please see our other Secondary Antibodies available on our website*
Streptavidin Alkaline Phosphatase (SA-AP)	R&D Systems Catalog # AR001, or equivalent
Wash Solution (TTBS)	50 mM Tris, 0.5 M NaCl, 0.05% Tween 20, pH 7.4
Blocking Buffer	3% BSA (Fraction V, 3x crystallized), TTBS, 0.2% azide, pH 7.4
Primary and Secondary Antibody Solution	0.1% BSA in TTBS, pH 7.4
Diluent Buffer	1% BSA (Fraction V, 3x crystallized), TTBS, 0.2% azide, pH 7.4
AP Substrate Buffer	0.1 M Tris, 0.1 M NaCl, 5 mM $MgCl_2$, pH 9.5
Color A	50 mg/mL nitro blue tetrazolium (NBT) in 70% dimethylformamide (DMF)
Color B	50 mg/mL bromochloroindolyl phosphate (BCIP) in 100% dimethylformamide (DMF)
Additional Tools	Forceps, clean plastic test tube, plastic trays, gloves, pipettes

Western Blot