Immunoblotting

Reviewed: Jan 15, 2023

Immunoblotting identifies proteins using antibodies that recognize unique epitopes in a linear amino acid sequence or folded structure. Typically, proteins are first separated by electrophoresis SOP 0007, then immobilized ("blotted") onto a membrane SOP 0008. This facilitates antibody recognition and allows for thorough washing to remove nonspecific interactions.
Detection is either direct or indirect as dye- or enzyme-conjugated secondary antibodies bind to the primary antibody and generate a signal proportional to the amount of epitope on the membrane. Antibodies from different host species (mouse, rabbit, goat, chicken) or subclasses (IgG1, IgG2a, etc.) can be combined for multiplexed probing. To detect multiple targets with same-species antibodies, membranes can be cut or stripped and reprobed several times. Fluorescent detection (*Alternative A*) is particularly suited for multiplexed quantitative imaging across a wide linear range. Chemiluminescent detection (*Alternative B*) offers higher sensitivity for low-abundance targets and can be performed with inexpensive homemade reagents, but the signal decays over minutes and is less suited to quantification.

This is a bench card. Full protocol available online.

Procedures

›› ***Immunoprobing***

- ☐ Incubation tray *or* Resealable plastic bag
- ☐ R0169 TBS with Tween™ 20, 1 L
- ☐ Bovine serum albumin *or* Nonfat dry milk powder ("Blotto")
- ☐ Antibodies, primary and secondary

(1.) Prepare blocking buffer by diluting Blotto to 5% or BSA to 1–5% in 20 mL TBST.

> ***Critical:*** Avoid milk-based blocking buffers such as Blotto for phospho-proteins or streptavidin-based detection. Milk contains phosphatases and biotin, respectively. Phosphatase activity can be quenched with 50 mM sodium fluoride.

(2.) Block the membrane face-up in 5 mL blocking buffer for 30 min at room temperature with agitation. 30 min

(3.) Dilute the primary antibody in blocking buffer (typically 1:1 000 to 1:10 000).

> ***Critical:*** For multiplexed experiments, avoid combining mouse and rat primary antibodies as they are difficult to cross-adsorb. Chicken antibodies tend to non-specifically bind to PVDF and nylon-based membranes, leading to high background.

(4.) Discard blocking buffer. Incubate with diluted primary antibody for 30–60 min at room temperature or overnight at 4 °C with agitation. 30–60 min

(5.) Transfer membrane to a clean tray.

(6.) Wash four times with 10–20 mL TBST for 3 min each with gentle shaking. 15 min

(7.) Dilute secondary antibody in TBST (typically 1:2 000 to 1:10 000) according to vendor instructions.

> ***Critical:*** Beware, secondary anti-mouse antibodies may be subclass-specific for IgG1, IgG2a, or IgG2b. Use highly cross-adsorbed secondaries when multiplexing with antibodies from different species.

(8.) Incubate with secondary antibody at room temperature for 30–60 min with agitation. 30–60 min

(9.) Wash again as before.

A › ***Fluorescent detection***

(1.) Specifications for infrared dyes used, for example, with LI-COR Odyssey® Imagers:

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- Use IRDye® 800CW or IRDye® 680RD secondary antibodies, at 1:20 000 dilution.
- Use IRDye® 800CW for the lowest-abundance target; it gives the lowest background.
- Rinse the membrane with 1 × TBS to remove residual Tween™ 20.

(2.) Clean the imager surface with distilled water, then 70% ethanol. Dry with a lint-free wipe.

(3.) Scan the membrane using a suitable fluorescence imager.

B › ***Luminescent detection***

- ☐ R0118 1 M Tris hydrochloride, 100 mL
- ☐ R0119 100 × Luminol, 10 mL
- ☐ R0120 200 × *p*-Coumaric acid, 10 mL
- ☐ R0121 ECL reagent, 10 mL

(1.) Prepare 10 mL ECL reagent per membrane just before use.

(2.) Drip-dry the membrane and distribute ECL reagent dropwise to cover the surface evenly. Incubate for 1 min at room temperature. 1 min

(3.) Briefly slip the membrane through deionized water and gently drag over the edge of a container to remove excess liquid.

(4.) Expose to film or camera. The signal will last for at least 10 min.

Stripping and reusing membranes

- ☐ R0122 Stripping buffer, 15 mL

(1.) Wash membrane in distilled water for 5 min.

(2.) Strip antibodies using one of the following:

Stripping buffer	NC	PVDF
0.2 M NaOH		
25 mM Glycine, pH 2.0		
Stripping buffer, pH 6.8	Not recommended	

Critical: Do not use high concentration of SDS with nitrocellulose membranes as this can destroy membrane integrity.

(3.) Wash membrane twice in distilled water for 5 min.

(4.) Reblock and reprobe as needed.

Recipe (available online) Troubleshooting (available online) Notes (available online)