TIPS, TRICKS & BEST PRACTICES

The Ultimate Western Blotting Guide

BUFFER PREPARATION

- Use freshly prepared buffers (pH can change during storage)
- Confirm pH, salt and detergent concentrations in:
  - **Sample loading and running buffer** to ensure consistent and uniform protein migration
  - **Transfer buffer** to ensure consistent and complete protein transfer from gel to membrane
  - **Blocking and antibody incubation buffers** for optimal antibody specificity and minimal background
- Include detergent in the blocking, wash, and antibody dilution buffers (eg. 0.05% Tween)
- Filter buffers through a 0.22µm or 0.45µm filter to avoid image artifacts from particulates and minimize microbial growth

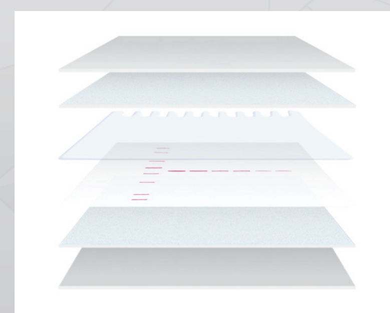
STEP 1: SAMPLE PREPARATION

- Optimize lysis conditions to fully solubilize target protein
  - Separate soluble from insoluble material by centrifugation after lysis
  - Determine protein concentration of the soluble fraction
  - Perform either a dot blot or a Western blot to confirm the presence of the target protein
    - Resolve an aliquot of the soluble fraction in one lane
    - Boil the insoluble pellet in sample buffer and resolve a fraction of the supernatant in a separate lane
  - Test several lysis protocols and detergents to identify the method that yields the most intense band in the soluble fraction relative to background when normalized for total protein load
- Include controls!
  - Positive control: Use a cell line extract that natively expresses the target protein
  - Negative control: Use a cell line extract from knockouts or treated samples that should not possess the target protein. Alternatively, perform the blot analysis without the primary antibody.

STEP 2: ELECTROPHORESIS — GELS

- Choose gel thickness:
  - 1mm gel thickness is typically ideal
  - Thicker gels allow for greater sample capacity
  - Thinner gels more efficiently transfer proteins
- Pick acrylamide percentage:
  - Use the lowest possible % of acrylamide to facilitate protein migration to the membrane during transfer
  - Choose a gradient gel for good resolution across a large molecular weight range

STEP 3: PROTEIN TRANSFER



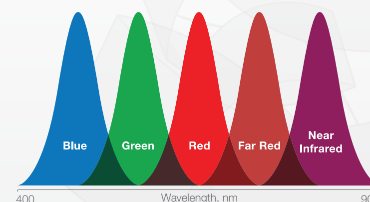
- Select the right membrane based on the detection chemistry:
  - **Chemiluminescence:**
    - PVDF is preferred over nitrocellulose because it has a has a higher protein binding capacity, which is especially important for detecting low abundance proteins
  - **Fluorescence:**
    - Low fluorescence PVDF is recommended because it has the lowest background across all wavelengths
    - Nitrocellulose works well for longer wavelengths (eg. 700/800 nm channels)
    - Standard PVDF should not be used because of its high background
  - Use a small pore size membrane (eg. 0.22µm) for small target proteins (<25kDa)
- Maximize contact between the gel and the membrane to ensure maximum protein transfer
- Pre-wet the membrane and filter paper before adding to the stack, use a roller during the assembly process to remove bubbles
- Confirm transfer efficiency, and measure the total protein in the gel before and after transfer
- Use a total protein stain on the membrane to confirm the absence of bubbles

STEP 4 : IMMUNODETECTION

- Determine the optimal concentration of primary and secondary antibodies that provides the best sensitivity within the linear range while minimizing background and non-specific binding
  - Test antibody titrations on well-characterized positive and negative samples
  - Test primary antibody concentrations between 1:500 – 1:2,000
    - An optimal concentration of primary antibody will give the highest signal within the linear range from binding of the target protein while minimizing non-specific binding
  - Test secondary antibody concentration 1:2,000 – 1:10,000
    - An optimal concentration of secondary antibody will provide strong signal without elevating background signal
- Optimize incubation conditions
  - Block the membrane to minimize non-specific binding of the antibodies
    - 1% casein usually works wells but if non-specific signal is too high, try other blocking buffers like BSA or fish gelatin
    - Rehydrated dry milk tends to create artifacts, avoid if possible
  - Test antibody incubation times
    - Short incubation times will compromise sensitivity
    - Long incubations can increase background

STEP 5: DETECT

- **Chemiluminescence detection:** Choose an ECL reagent that provides sufficient sensitivity for your application
- **Fluorescence detection:** Select fluorochromes with optimal excitation and emission wavelengths to minimize cross-talk between channels
  - Low abundance proteins: Choose fluorochromes which emit in the far red/near IR for the best sensitivity (signal to noise ratio)
  - High abundance proteins: Choose fluorochromes in the green/blue channels (i.e. housekeeping proteins)
- Optimize the exposure time to eliminate saturated pixels while maintaining a large dynamic range to detect both bright and faint bands.



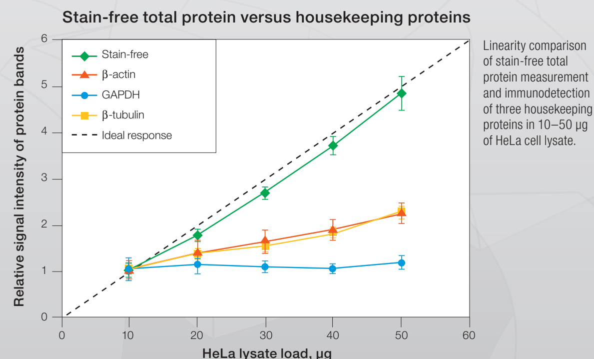
STEP 6: IMAGING

- Choose a high quality digital imaging system for the best balance between sensitivity, dynamic range and accuracy of quantitation
  - CCD cameras usually have a better linear range than film
- Use "binning" to adjust the balance between sensitivity and resolution
  - High settings (4×4, 6×6, 8×8): Provide the best sensitivity and detection of faint bands
  - Low settings (1×1, 2×2, 3×3): Ideal for imaging abundant target proteins

STEP 7: ANALYSIS

- Define the linear dynamic range in which signal intensity is directly proportional to the protein quantity
  - Resolve serial dilutions of a known sample, transfer to a membrane and blot using optimal conditions
  - Determine the range in which a 1:1 relationship between sample concentration and signal is retained
- Normalize target protein to total lane protein
  - Total protein is a more accurate loading control because it does not depend on the expression of a single protein remaining stable across all samples, and has a signal-to-protein ratio closer to 1:1 over a broader range of protein loads
- Use multiplex fluorescent Western blot if using housekeeping proteins
  - Avoids inaccuracies that result from extra blot manipulation by eliminating the need to strip and re-probe
  - Validate the accuracy of housekeeping proteins using a total protein stain to normalize, or normalize your protein of interest to total lane protein
- Check out this resource for more: **bio-rad.com/webroot/web/pdf/lsr/literature/RP0058.pdf**

Stain-free total protein versus housekeeping proteins



Linearity comparison of stain-free total protein measurement and immunodetection of three housekeeping proteins in 10–50 µg of HeLa cell lysate.

Review our Protein Blotting Guide for even more tips & tricks! **bio-rad.com/webroot/web/pdf/lsr/literature/Bulletin_2895.pdf**

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