

# 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 (for example, 0.05% Tween)
- Filter buffers through a 0.22 or 0.45  $\mu\text{m}$  filter to avoid image artifacts from particulates and to minimize microbial growth

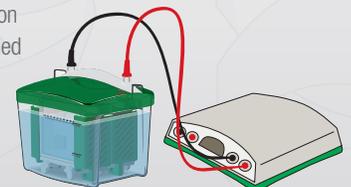


## STEP 1: SAMPLE PREPARATION

- **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

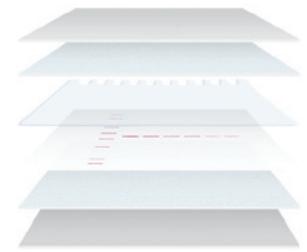
## STEP 2: ELECTROPHORESIS

- Choose gel thickness
  - ♦ 1 mm gel thickness is typically ideal. Thicker gels allow for greater sample capacity while thinner gels more efficiently transfer proteins
- Pick acrylamide percentage
  - ♦ Use the lowest possible percentage of acrylamide to facilitate protein migration to the membrane during transfer
  - ♦ Choose a gradient gel for good resolution across a large molecular weight range
    - For new or unknown samples, first try a broad gradient, such as 4–20% or 8–16%, or Any kD gel for a global evaluation of the sample. Then use an appropriate single-percentage gel once a particular size range of proteins has been identified
- Choose a **Stain-Free Precast Gel**
  - ♦ Retains Laemmli-like separation characteristics using standard sample and Tris/glycine running buffers
    - Gel images and complete analysis in less than 5 minutes after electrophoresis
    - Comparable sensitivity to Coomassie stain
    - Stain-Free imaging allows for the normalization of bands to the total protein on a blot, eliminating the use of housekeeping proteins or the need for stripping and reprobing
- Select protein standards that offer good resolution of the proteins in the size range of interest and compatibility with downstream analysis (for example, blotting)
- **Power supplies** hold one parameter constant (either voltage, current, or power) during electrophoresis. However, the resistance does not remain constant
  - ♦ Constant voltage: preferred when running multiple gels in parallel
  - ♦ Constant current: results in shorter but hotter runs than constant voltage runs. Heat increases protein diffusion and leads to fuzzier bands unless additional cooling is supplied
  - ♦ Constant power: minimizes the risk of overheating
- Visit the [Stain-Free Western Blotting page](#) to learn more about Stain-Free technology



### STEP 3: PROTEIN TRANSFER

- Select the right membrane based on the detection chemistry
  - ◆ Chemiluminescence
    - **PVDF** is preferred over nitrocellulose because it 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 (for example, 700/800 nm channels)
    - Standard PVDF should not be used because of its high background
  - ◆ Use a small pore size membrane (for example, 0.22  $\mu\text{m}$ ) for small target proteins (<25 kD)
- Maximize contact between the gel and the membrane to ensure maximum protein transfer by using a **roller** during the assembly process to remove bubbles
  - ◆ Freshly prepare transfer buffer
- If using dry membranes and filter paper, prewet before assembling the transfer stack
- Use a **Stain-Free enabled imager** or a total protein stain on the membrane to confirm the complete transfer of proteins to the membrane from the gel pre-and posttransfer



### STEP 4: IMMUNODETECTION

- Determine the optimal concentration of primary and secondary antibodies that provide the best sensitivity within the linear range while minimizing background nonspecific binding
  - ◆ Use high-quality primary antibodies
    - A good antibody is sensitive, meaning it can detect low amounts of your target, and is also specific, recognizing only the target without giving spurious secondary bands
  - ◆ Test antibody titrations on well-characterized positive and negative samples
  - ◆ Test primary antibody concentrations 1:500–1:2,000
    - An optimal concentration of primary antibody will give the highest signal within the linear range
  - ◆ Test secondary antibody concentration 1:2,000–1:10,000
    - An optimal concentration of secondary antibody will provide strong signal without elevating background signal
    - Use cross-adsorbed secondary antibodies that offer a lower chance of cross-reactivity and reduced background, giving more reliable results
- Optimize **incubation conditions**
  - ◆ Block the membrane to minimize nonspecific binding of the antibodies
    - Use a versatile blocking buffer that works for chemiluminescence and fluorescence detection such as **EveryBlot Blocking Buffer** to reduce background
    - Another option is **1% Casein** in PBS or TBS, but if nonspecific binding is still too high, try other blocking buffers such as BSA or fish gelatin, or try adding a mild detergent (for example, 0.1% Tween 20)
    - Rehydrated dry milk tends to create artifacts; avoid if possible
  - ◆ Test antibody incubation times
    - Short incubation times will compromise sensitivity
    - Long incubation times can increase background
- Chemiluminescence detection
  - ◆ Choose an ECL reagent such as **Clarity™** or **Clarity Max Western ECL Substrate** 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 that emit in the far red/near infrared for the best sensitivity (signal-to-noise ratio)
    - High-abundance proteins: choose fluorochromes in the green/blue channels (for example, housekeeping proteins)
    - Try **StarBright™ Blue Fluorescent Secondary Antibodies** conjugated to a high-yield fluorophore for ultra-sensitive fluorescence detection with very low background of single or multiple proteins
  - ◆ Optimize the exposure time to eliminate saturated pixels while maintaining a large dynamic range to detect both bright and faint bands
  - ◆ Always use a **rocker** for incubation and washing steps. Consistent agitation ensures even distribution of wash buffer and antibodies, preventing uneven binding or washing across the membrane that could lead to nonspecific binding and high background

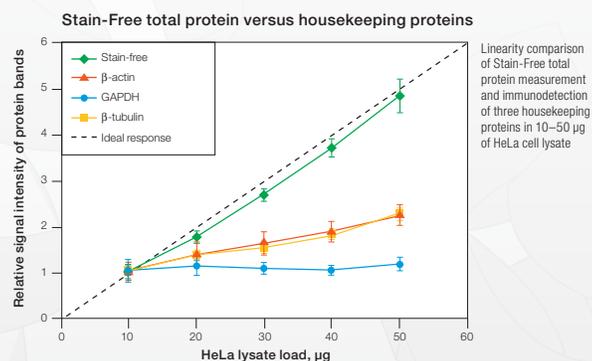


## STEP 5: IMAGE ACQUISITION

- Choose a high-quality digital imaging system such as the **ChemiDoc™** or **GelDoc™** Imaging Systems for the best balance among sensitivity, dynamic range, and accuracy of quantitation
  - Imagers with CMOS or 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
- Turn on your imager's "show saturated pixels" option. This helps you avoid excessively long exposure times that lead to saturated bands and unquantifiable data
- Molecular weight standards are often very intense, causing auto-exposure algorithms to take exposures too short for your proteins of interest. Either manually enter a longer exposure time or, if using a Bio-Rad imager, choose the "region-of-interest" feature to specify the area of the blot to apply the auto-exposure algorithm

## STEP 6: IMAGE ANALYSIS

- Choose an imaging software such as **Image Lab Software** that allows you to easily acquire and analyze robust, publishable results
  - The Image Lab Software, Security Edition, package helps enable U.S. FDA CFR 21 Part 11 compliance
- 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
  - Choose an imager that uses extended dynamic range imaging, such as the ChemiDoc systems, in order to capture blots that contain both very faint and very intense bands
- Normalize target protein to total lane protein
  - Total protein normalization (TPN) 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
  - TPN takes into account intensity of all proteins in the lane, sample loading variations, and variations during electrophoresis and transfer
  - Meets **JBC's guidelines** for collecting and presenting data in publications
- Use multiplex fluorescent western blotting if using housekeeping proteins
  - Multiplex fluorescent western blotting avoids inaccuracies that result from extra blot manipulation by eliminating the need to strip and reprobe
- Learn more about [quantifiable western blot analysis](#)



Find more tips and tricks in the [Western Blot Learning Center](#).

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