# General Stain-Free Western Blotting

Protocol

**Abstract**

Western blotting is a widely adopted, powerful technique to separate and detect proteins, estimate their quantities, and determine their molecular weights. Proteins of interest are extracted from cells or tissues and processed in a multistep western blotting workflow. This protocol describes Bio-Rad's Stain-Free Western Blotting Workflow, which takes only 5 hours to complete versus 16 hours for traditional western blotting. When following the lengthy and laborious traditional western blotting workflow, researchers assess the adequacy of their western blot data at the final step of the procedure. Stain-Free technology enables users to determine transfer efficiency and collect additional data quality information quickly and easily throughout the western blotting process.

## Procedure for Chemiluminescent Western Blotting

### 1 Protein Sample Preparation for Western Blot Analysis

1. Place the cell culture dish on ice and wash the cells with ice-cold Tris-buffered saline (TBS; 20 mM Tris, pH 7.5, 150 mM NaCl).
2. Aspirate the TBS, then add 1 ml per 100 mm dish ice-cold RIPA buffer (50 mM Tris-HCl [pH 8.0], 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, 1 mM NaF, 1 mM sodium orthovanadate, Roche Protease Inhibitor Tablet).
3. Scrape adherent cells off the dish using a cold plastic cell scraper and gently transfer the cell suspension into a precooled microcentrifuge tube.
4. Maintain constant agitation for 30 min at 4°C.
5. If necessary, sonicate three times for 10–15 sec to complete cell lysis and shear DNA to reduce sample viscosity.
6. Spin at 16,000 x g for 20 min in a 4°C precooled centrifuge.
7. Gently remove the centrifuge tube and place it on ice. Transfer the supernatant to a fresh tube, also kept on ice, and discard the pellet.
8. Remove a small volume (10–20 µl) of lysate to perform a protein assay. Determine the protein concentration for each sample.
9. If necessary, aliquot the protein samples for long-term storage at –20°C. Repeated freeze-thaw cycles cause protein degradation and should be avoided.
10. Take 20 µg of each sample and add an equal volume of 2x Laemmli sample buffer.
11. Boil each cell lysate in sample buffer at 95°C for 5 min.
12. Centrifuge at 16,000 x g in a microcentrifuge for 1 min.

### 2 Electrophoresis with Stain-Free Gels (~30 min)

1. Take an Any kD Criterion TGX Stain-Free Precast Gel, remove the comb, and remove the tape from the bottom of the cassette.

**Note:** Gel percentage selection depends on the size of the protein of interest. A 4–20% gradient gel separates proteins of all sizes very well. It also facilitates better transfer of proteins of all sizes.

2. Place the cassette in a Criterion Cell and fill each integrated upper buffer chamber with 60 ml 1x Tris/Glycine/SDS running buffer (dilute from 10x TGS, 192 mM glycine, 0.1% SDS, pH 8.3).
3. Fill each half of the lower buffer tank with 400 ml running buffer to the marked fill line.
4. Load the protein samples (10–20 µg each) and protein standard.

**Note:** We recommend using Precision Plus Protein Unstained Standards with TGX Stain-Free Gels and Precision Plus Protein All Blue Standards with fluorescent western blots.

5. Place the lid on the tank, aligning the color-coded banana plugs with corresponding jacks on the lid.
6. Set the voltage to 200–300 V and run the gel for 20–30 min.

**Note:** Midi format gels take approximately 30 min; mini format gels take only 15–20 min.

### ❸ Visualize Separation Using the ChemiDoc™ Go or ChemiDoc MP Imaging System (~5 min)

1. After electrophoresis is complete, turn off the power supply and disconnect the electrical leads.
2. Remove the gel cassette from the cell. Pull the two plates of the cassette apart to expose the gel using the Mini-PROTEAN™ Cassette Opening Lever.
3. Carefully lift the gel from the cassette and put it on the sample stage of the ChemiDoc Go or ChemiDoc MP Imaging System.
4. Capture the Stain-Free gel image (image A) using the following settings:
   - Application: Stain-Free Gel
   - Gel activation time: 45 sec
   - Image Size (W x L): medium; 15.5 x 12.4 cm
   - Exposure: optimal automatic exposure
5. Remove the gel from the sample stage and keep it wet in running buffer.

### ❹ Protein Transfer with the Trans-Blot™ Turbo System (~10 min)

1. Open a Trans-Blot Turbo Midi Nitrocellulose Transfer Pack and place the pad with the membrane on the base of the transfer cassette.
2. Place the gel on top of the membrane, place the top pad on the gel, and roll out bubbles.
3. Place the lid on the cassette base and lock it.
4. Insert the cassette into either instrument bay.
5. Start the transfer by selecting the preset Turbo program and choosing the Criterion Gel size, and then press **RUN**. For most proteins, the 7 min protocol is ideal.
6. When the transfer is complete, disassemble the blotting sandwich and place both the blot and the gel in a container with deionized water.

### ❺ Verify Transfer Using the ChemiDoc Go or ChemiDoc MP Imaging System (~5 min)

1. Put the post-transfer gel on the sample stage of the ChemiDoc Go or ChemiDoc MP Imaging System.
2. Capture the Stain-Free image of the post-transfer gel (image B) with the following settings:
   - Application: Stain-Free Gel
   - Gel activation time: none
   - Image Size (W x L): medium; 15.5 x 12.4 cm
   - Exposure: same as the exposure time for the pre-transfer gel image

**Note:** This Stain-Free image of the post-transfer gel is used to verify the transfer efficiency.

3. Remove the gel from the sample stage and image the blot with the following settings:
   - Application: Stain-Free Blot
   - Image Size (W x L): medium; 15.5 x 12.4 cm
   - Exposure: optimal automatic exposure

**Note:** Keep the blot wet all the time; do not let it dry. This Stain-Free blot image is used to check the transfer quality.

4. Remove the blotting membrane from the sample stage and place it in a container with 1x TBS + 0.1% Tween-20 (TBST).

### ❻ Antibody Incubation (~4 hr)

1. Block in EveryBlot Blocking Buffer for 5 min at room temperature with agitation.
2. Dilute the primary antibody in full-strength EveryBlot Blocking Buffer and incubate the membrane for 1 hr at room temperature with agitation.

**Note:** The antibody should be diluted in the blocking buffer according to the manufacturer's recommended ratio. Primary antibody may also be applied to the blot overnight at 4°C, depending on the antibody quality and performance.

3. Rinse the blot five times for 5 min with TBST.
4. Dilute the horseradish peroxidase (HRP)–conjugated secondary antibody in full-strength EveryBlot Blocking Buffer and incubate the membrane for 1 hr at room temperature with agitation.

**Note:** As an alternative to EveryBlot Blocking Buffer, the antibody can be diluted in 1% casein or 5% skim milk in TBS at the manufacturer's recommended ratio. However, blocking times will vary.

5. Rinse the blot five times for 5 min with TBST.

### ❼ Imaging and Analysis Using the ChemiDoc Go or ChemiDoc MP Imaging System and Image Lab Software (~10 min)

1. After antibody incubation, place the blot back on the sample stage of the ChemiDoc Go or ChemiDoc MP Imaging System and capture a Stain-Free image of the blot (image C) with the following settings:
   - Application: Stain-Free Blot
   - Image Size (W x L): medium; 15.5 x 12.4 cm
   - Exposure: optimal automatic exposure

**Note:** Keep the blot wet all the time; do not let it dry. This Stain-Free blot image is used for total protein loading control and normalization.

2. After imaging, keep the membrane in TBST while preparing the Clarity™ Max Western ECL Substrate mixture.
3. Mix the Clarity Max Substrate Kit components in a 1:1 ratio. Prepare 0.02 ml of solution/cm$^2$ of membrane. For a midi-sized membrane (8.5 x 13.5 cm), approximately 2 ml of solution is sufficient.
4. Incubate the membrane in the substrate solution for 5 min.
5. Place the blotting membrane back on the sample stage of the ChemiDoc Go or ChemiDoc MP Imaging System.
6. Capture the chemiluminescent signals on the blot (image D) with the following settings:
   - Application: Chemiluminescent Blot
   - Image Size (W x L): medium; 15.5 x 12.4 cm
   - Exposure: optimal automatic exposure

**Note:** The Image Size for the Stain-Free and chemiluminescence images must be the same for follow-up total protein normalization by Image Lab Software.

#### Validate Total Protein Normalization (~5 min)

1. Download the chemiluminescence blot image of the protein of interest and the Stain-Free blot image captured in the imaging step and open them in Image Lab Software.
2. Link the chemiluminescence and Stain-Free images for normalization analysis by creating a multichannel image.
   - Select **Create Multichannel Image** from the File dropdown menu
   - Drag the chemiluminescence and Stain-Free blot images into channels 1 and 2

**Note:** The two images may not align perfectly.

   - Click **OK**
   - Hide the multichannel overlay and chemiluminescence channel by deselecting them from the horizontal toolbar (located at the top of the image)
3. Select and use **Detect Lanes and Bands** tools to detect lanes in the Stain-Free image.

**Note:** Although unnecessary for total protein normalization, bands can also be defined if a protein standard is present. Define bands only in the protein standard lane.

4. Define the normalization channel.
   - In the normalization section under Lanes and Bands, set the normalization channel to Stain-Free Blot
   - Define a normalization lane

**Note:** This lane should not contain a protein standard.

   - Show the chemiluminescence image by selecting it from the horizontal toolbar
   - Select **Sync Normalization Lanes** to populate lanes on the chemiluminescence image and make adjustments if necessary
5. Detect the bands of interest in the chemiluminescence image.
6. To view the normalized target protein volumes, click **Analysis Table** on the toolbar. All calculations will be performed automatically by the software, including the Normalization Factor and Normalized Volume. The target protein band intensity values are now adjusted for variation in the protein load. This will allow for accurate comparison of target proteins among the samples.

## Procedure for Multiplexing Fluorescent Western Blotting

Follow the steps described in the Procedure for Chemiluminescent Western Blotting section for sample preparation, electrophoresis, protein transfer, transfer verification, and antibody transfer. Variation in protocols between the chemiluminescent and multiplex fluorescent western blotting procedures will start at the secondary antibody incubation step.

#### ❶ Antibody Incubation

Follow the instructions in steps 1–5 in the Antibody Incubation section on page 2.

1. Dilute the appropriate secondary antibodies, StarBright™ 520 and StarBright 700, to match the species of the primary antibodies, in full-strength EveryBlot Blocking Buffer and incubate the membrane for 1 hr at room temperature with agitation.

**Note:** If EveryBlot Blocking Buffer was not used, the antibodies can be diluted in 1% casein or 5% skim milk in TBS according to the manufacturer's recommended ratios. Selection of commercially available secondary antibodies should be based on the species of the primary antibodies and the fluorescence compatibility with the imaging system.

2. Rinse the blot five times for 5 min with TBST.

**❷ Imaging and Analysis by Image Lab Software — Total Protein Normalization (~5 min)**

1. Make the following selections to acquire a three-channel multiplex fluorescence image of the blot (image E).

   Channel 1:
   - Application: StarBright 520 Blot
   - Image Size (W x L): medium; 15.5 x 12.4 cm
   - Exposure: optimal automatic exposure

   Channel 2:
   - Application: StarBright 700 Blot
   - Image Size (W x L): medium; 15.5 x 12.4 cm
   - Exposure: optimal automatic exposure

   Channel 3 (optional):
   - Application: Far Red or Near-Infrared (FR/NIR) Blot (DyLight 680 or 800)
   - Image Size (W x L): medium; 15.5 x 12.4 cm
   - Exposure: optimal automatic exposure

2. Download the multichannel image captured on the ChemiDoc Go or ChemiDoc MP Imaging System and open it in Image Lab Software. Hide the multichannel overlay and fluorescence channels by deselecting them from the horizontal toolbar (located at the top of the image).

3. Select and use **Lane and Bands tools** to detect lanes for the Stain-Free image.

**Note:** Although unnecessary for total protein normalization, bands can also be defined if a protein standard is present. Define bands only in the protein standard lane.

4. Define the normalization channel.
   - In the normalization section under Lanes and Bands, set the normalization channel to Stain-Free Blot
   - Define a normalization lane

**Note:** This lane should not contain a protein standard.
   - Show the fluorescence channel images by selecting them from the horizontal toolbar
   - Press **Sync Normalization Lanes** to populate lanes on the fluorescence channel images and make adjustments if necessary

5. Detect the bands of interest in the fluorescence channel images.

6. To view the normalized target protein volumes, click **Analysis Table** on the toolbar. All calculations will be performed automatically by the software, including the Normalization Factor and Normalized Volume. The target protein band intensity values are now adjusted for variation in the protein load. This will allow for accurate comparison of target proteins among the samples.

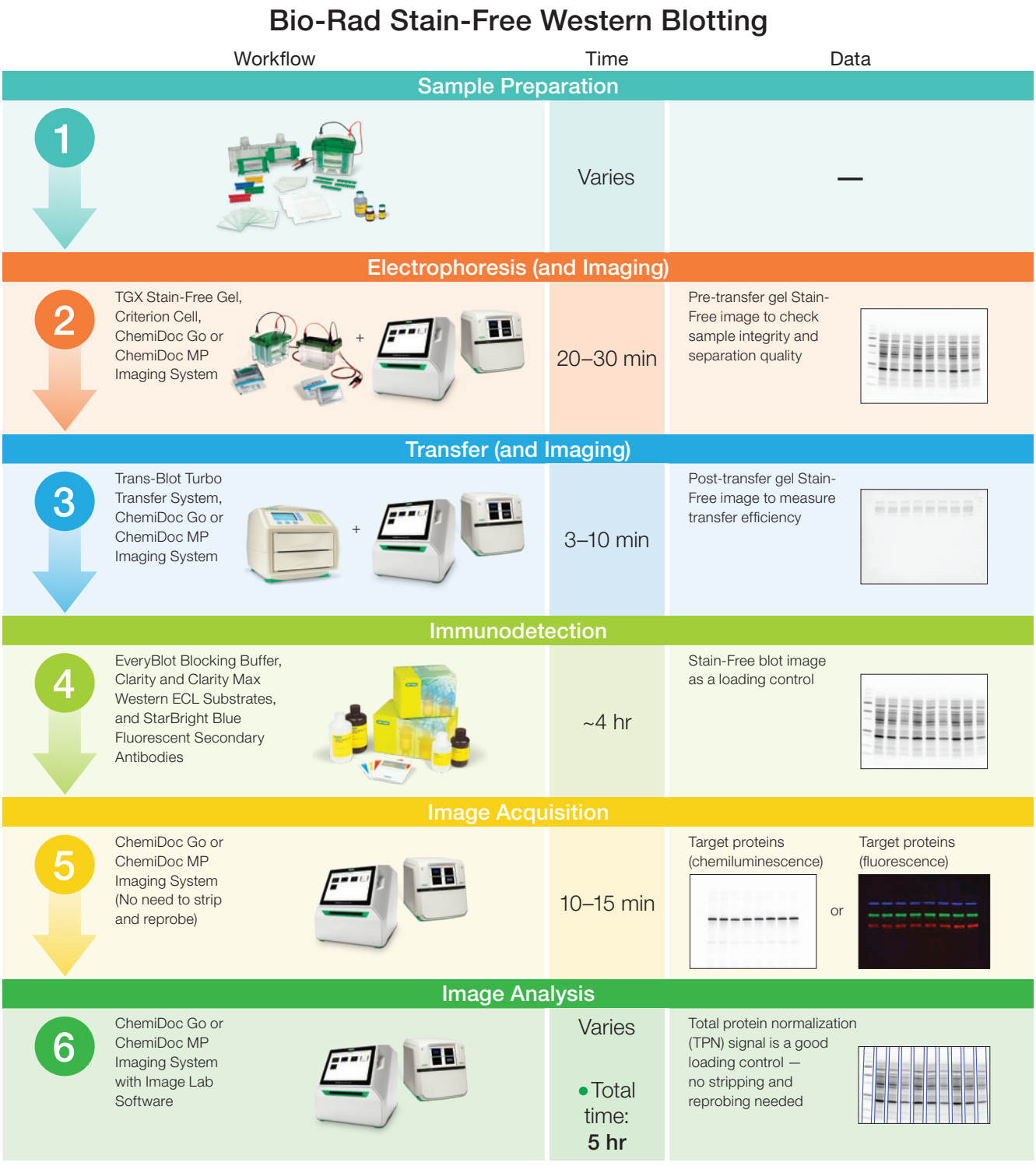


**Fig. 1. Bio-Rad Stain-Free western blotting workflow steps and example images.** Workflow instruments, reagents, and processing times are summarized as shown in the figure. Images in steps 2–6 were generated in a multiplex fluorescent western blotting experiment. In this experiment, AKR1C2 was probed in eight replicates of HeLa lysate (10 µg per lane) using a mouse antibody (Bio-Rad Laboratories, Inc., #VMA00346, 1:1,000) and a DyLight 800–conjugated goat anti-mouse antibody (Bio-Rad, #STAR117D800GA, 1:15,000). Protein ATG7 was probed using a rabbit antibody (Bio-Rad, #12004161, 1:2,500) and StarBright Blue 700 Goat Anti-Rabbit antibody (Bio-Rad, #STAR36D680GA, 1:15,000). A housekeeping protein, ß-tubulin, was probed using the hFAB Rhodamine Anti-Tubulin IgG primary antibody (Bio-Rad, #12004165, 1:1000). A high-percentage gel was intentionally used in this experiment to show that some large proteins remained in the gel after transfer (step 3). A gradient gel (for example, 4–15%) should be used for better transfer efficiency if the target protein size is more than 100 kD. The chemiluminescent blot in step 5 was generated from a different experiment where ß-tubulin was probed in HeLa cell lysate at different loads from 10 to 50 µg, using a mouse monoclonal ß-tubulin antibody (Rockland Immunochemicals, Inc., 1:4,000) and an HRP-conjugated goat anti-mouse antibody (Jackson ImmunoResearch Laboratories, Inc., 1:50,000).

## Bio-Rad Stain-Free Western Blotting Workflow Overview

In Figure 1 above, the Stain-Free western blotting workflow is depicted in the left column in six steps. The major instruments and reagents used in the workflow are shown at each step. The estimated time for each step is also included. When performing Stain-Free western blotting, stripping and reprobing the blot for housekeeping proteins is not needed. The Stain-Free blot is a suitable loading control.

The right column of the figure shows that a minimum of four images can be generated in the Stain-Free western blotting workflow. The use of each piece of data is described. The Stain-Free images of the pre-transfer gel, post-transfer gel, and blot (Figure 1, steps 2–4) cannot be generated in such a convenient and reliable way if using a traditional approach, but they provide important information and checkpoints along the way that improve control and reproducibility of the western blot workflow.

As shown in step 5, the target protein signals can be captured either on a chemiluminescence blot image, if an HRP-conjugated secondary antibody was applied in detection, or on a fluorescence blot image, if multiplex fluorescent western blotting was performed, to detect more than one target protein simultaneously on the same blot.

Reliable assessment of changes in target protein expression levels requires measuring both the target protein and the loading control protein in their linear dynamic ranges for immunodetection. Traditionally, housekeeping proteins (HKP) such as actin, β-tubulin, or GAPDH have served as controls for loading and data normalization. Unfortunately, HKP are usually highly expressed, whereas target proteins are often expressed in low abundance. Thus, HKP can yield over-saturated reference bands that are out of their linear range. HKP expression levels may not be constant but instead vary with different experimental treatments and other factors. Stain-Free technology eliminates these issues by quantitating total proteins on the same blot as the proteins of interest. This method of data normalization, called total protein normalization (TPN), provides more accurate protein quantitation and western blotting results than traditional HKP approaches.

Visit **bio-rad.com/StainFreeWestern** for more information.



TGX Stain-Free Precast Gels are covered by U.S. Patent Numbers 7,569,130 and 8,007,646. Clarity Max Western ECL Substrate is manufactured by Cyanagen Srl and is the subject of patent application numbers US7855287, EP1950207, US9040252, AU2011202658, CA2742025, US8129136, and EP1962095, together with other equivalent granted patents and patent applications in other countries like CN102313732.

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