General Stain-Free Western Blotting Protocol

Protocol

Abstract

Western blotting is a very useful and widely adopted laboratory technique, but the traditional procedure can be long and tedious. It is only at the end of the long procedure that a researcher can assess whether the western blot data are inadequate due to poor loading controls. Bio-Rad has developed Stain-Free western blotting to address the major concerns associated with the traditional western blotting procedure. This protocol describes the Stain-Free western blotting workflow. It enables faster, quantitative, and more transparent and reliable western blotting.

Procedure for Chemiluminescent Western Blotting

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.

1 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 running buffer (25 mM Tris, 190 mM glycine, 0.1% SDS, pH 8.3). Rinse the wells with running buffer.
3. Fill each half of the lower buffer tank with 400 ml running buffer to the marked fill line.
4. Load the protein samples (20 µg each) and protein standard.

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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.

Visualize Separation Using the 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.
3. Carefully lift the gel from the cassette and put it on the sample stage of the 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
   - Imaging size (W x L): medium; 15.5 x 12.4 cm
   - Image exposure: optimal automatic exposure
5. Remove the gel from the sample stage and keep it wet in running buffer.

2 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 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 over, disassemble the blotting sandwich and place both the blot and the gel in a container with deionized water.

Verify Transfer Using the ChemiDoc MP Imaging System (~5 min)

1. Put the post-transfer gel on the sample stage of the 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
   - Imaging size (W x L): medium; 15.5 x 12.4 cm
   - Image exposure time: 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
   - Imaging size (W x L): medium; 15.5 x 12.4 cm
   - Image 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 TBS with Tween 20 (TBST).

3 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 six times for 5 min with TBST.

4 Imaging and Analysis Using the 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 MP Imaging System and capture a Stain-Free image of the blot (image C) with the following settings:
   - Application: Stain-Free Blot
   - Imaging size (W x L): medium; 15.5 x 12.4 cm
   - Image 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 0.1 ml of solution/cm^2 of membrane. For a midi-sized membrane (8.5 x 13.5 cm), 12 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 MP Imaging System.
6. Capture the chemiluminescent signals on the blot (image D) with the following settings:
   - Application: Chemiluminescent Blot
   - Imaging size (W x L): medium; 15.5 x 12.4 cm
   - Image exposure: optimal automatic exposure

Note: The image area for the Stain-Free and chemiluminescent images must be the same for follow-up total protein normalization by Image Lab Software.

Validate Total Protein Normalization (~5 min)

1. Download the chemiluminescent 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 chemiluminescent and Stain-Free images for normalization analysis by creating a multichannel image.
   - Select **Create Multichannel Image** from the File dropdown menu
   - Drag the chemiluminescent and Stain-Free blot images into channels 1 and 2

Note: The two images may not align perfectly.

   - Press **OK**
   - Hide the multichannel overlay and chemiluminescent channel 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 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 chemiluminescent image by selecting it from the horizontal toolbar
   - Press **Sync Normalization Lanes** to populate lanes on the chemiluminescent image and make adjustments if necessary
5. Detect the bands of interest in the chemiluminescent 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

Please 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 multiplexing fluorescent western blotting procedures will start at the secondary antibody incubation step.

3 Antibody Incubation

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

1. Dilute the secondary antibodies, DyLight 680 conjugated to goat anti-mouse and DyLight 800 conjugated to goat anti-rabbit, 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 in steps 1–5, 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 six times for 5 min with TBST.

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

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

   Channel 1:
   - Application: DyLight 680 Blot
   - Imaging size (W x L): medium; 15.5 x 12.4 cm
   - Image exposure: optimal automatic exposure

   Channel 2:
   - Application: DyLight 800 Blot
   - Imaging size (W x L): medium; 15.5 x 12.4 cm
   - Image exposure: optimal automatic exposure

   Channel 3:
   - Application: Stain-Free Blot
   - Imaging size (W x L): medium; 15.5 x 12.4 cm
   - Image exposure time: optimal automatic exposure

2. Download the multichannel image captured on the ChemiDoc MP Imaging System and open it in Image Lab Software. Hide the multichannel overlay and fluorescent 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.

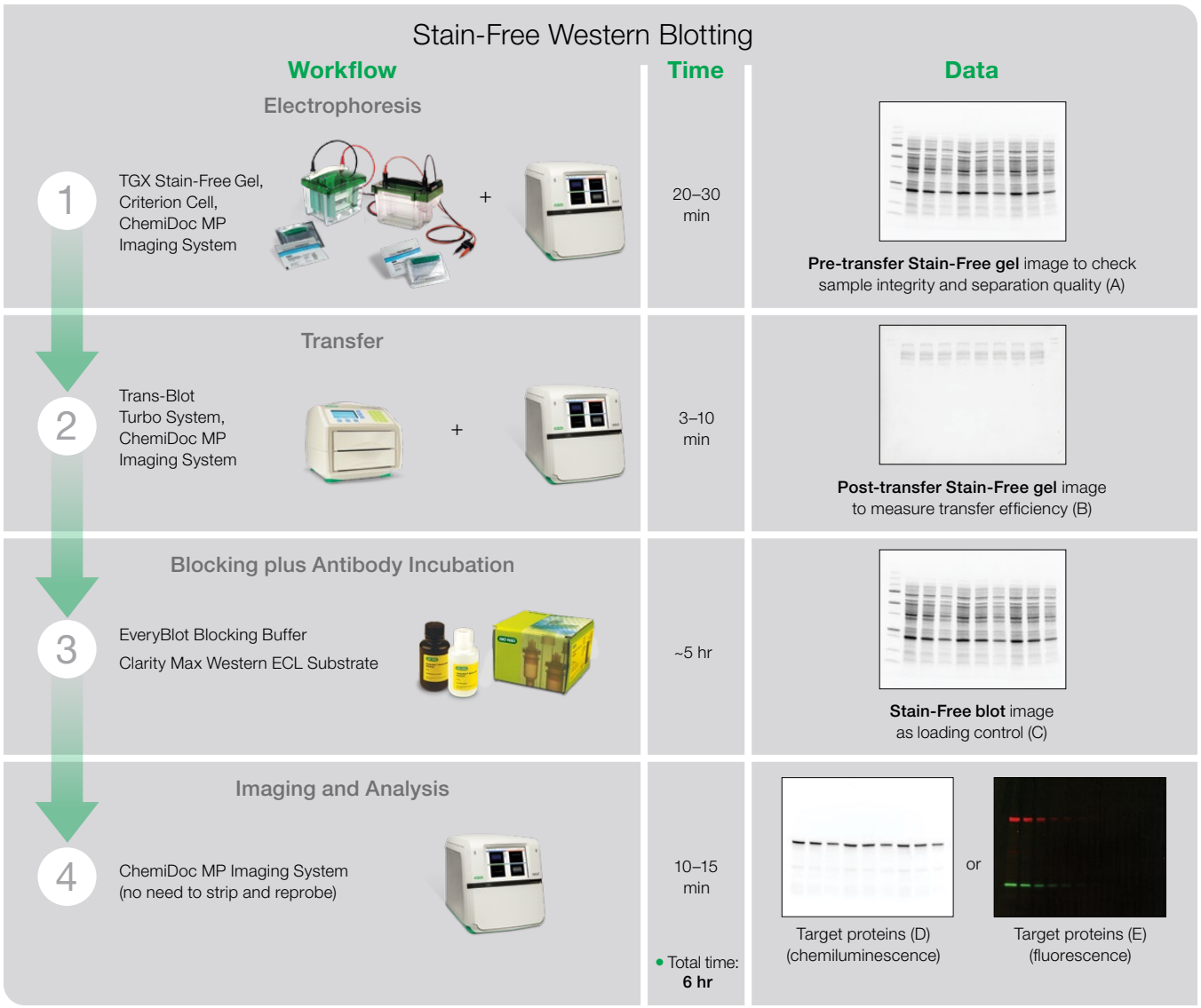
Note: Although unnecessary for total protein normalization, bands can also be defined if a protein standard is present. Define bands only in the protein standard lane.

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 fluorescent channel images by selecting them from the horizontal toolbar
   - Press **Sync Normalization Lanes** to populate lanes on the fluorescent channel images and make adjustments if necessary

5. Detect the bands of interest in the fluorescent 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.



Bio-Rad Stain-Free Western Blotting

The Stain-Free western blotting workflow is depicted in the left column in four 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 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 (A, B, C) 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.

The target protein signals can be captured either on a chemiluminescent blot image (D), if an HRP-conjugated secondary antibody was applied in detection, or on a fluorescent blot image (E), if multiplexing fluorescent western blotting was performed, to detect more than one target protein simultaneously on the same blot (E).

Images A, B, C, and E were generated in a multiplex fluorescent western blotting experiment. In this experiment, BCL2 was probed in a 2x dilution series of Jurkat cell lysate (starting at 10 µg) using a mouse antibody (Bio-Rad Laboratories, Inc., VMA00017, 1:1,000) and a DyLight 800–conjugated goat anti-mouse antibody (Bio-Rad, STAR117D800GA, 1:15,000). Protein UBA1 was probed using a rabbit antibody (Bio-Rad, VPA00195, 1:1,000) and a DyLight 680–conjugated sheep anti-rabbit antibody (Bio-Rad, STAR36D680GA, 1:15,000). A high-percentage gel was intentionally used in this experiment to show that some large proteins remained in the gel after transfer (B). A gradient gel (for example, 4–15%) should be used for better transfer efficiency if the target protein size is more than 100 kD.

Image D 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).

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

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