General V3 Western Workflow™ Blotting Protocol

Bulletin 6390

Protocol

Western blotting is a very useful and widely adopted lab technique, but the traditional procedure can be long and tedious. Researchers can assess whether a blot image is captured only at the end of the long procedure and the quality of western blot data is sometimes inadequate due to poor loading controls. Bio-Rad has developed the V3 Western Workflow utilizing stain-free technology to address the major concerns associated with the traditional western blotting procedure. This protocol describes the V3 Western Workflow affording faster, more transparent, quantitative, and more reliable western blotting.

Procedure for Chemiluminescent Western Blotting

Protocol

Protein sample preparation for western blot analysis

1. Place the cell culture dish in 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 pre-cooled 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 a Criterion™ TGX Any kD Stain-Free™ precast gel, remove the comb, and tape from the bottom of the cassette.

   Note: Gel percentage selection is dependent on the size of the protein of interest. A 4–20% gradient gel separates the proteins of all sizes very well. It also facilitates better protein transfer 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 markers.
5. Place the lid on the tank, aligning the color-coded banana plugs with corresponding jacks on the lid.
6. Run the gel for 5 min at 50 V.
7. Increase the voltage to 200–300 V to finish the run in about 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 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 imager.

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4. Start Image Lab™ software and capture the stain-free gel image (Image A) using the following settings:
   - Application: Stain-Free gel
   - Gel activation time: 1 min
   - Imaging area: Bio-Rad Criterion gel
   - Image exposure time: automatically optimized for the most intense bands
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 PVDF 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. A typical run takes only 7 min.
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 system (~5 min)

1. Put the post-transfer gel on the sample stage of the ChemiDoc MP imager.
2. Start Image Lab software and 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 area: Bio-Rad Criterion gel
   - 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 area: Bio-Rad Criterion gel
   - Image exposure time: automatically optimized for the most intense bands

   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 TBST.

3 Antibody incubation (~5 hr)

1. Block in 3% bovine serum albumin (BSA) in TBST at room temperature for 1 hr.
2. Incubate overnight in the primary antibody solution (against the target protein) at 4°C.

   Note: The antibody should be diluted in the blocking buffer according to the manufacturer's recommended ratio. Primary antibody may be applied to the blot for 1–3 hr at room temperature, depending on the antibody quality and performance.
3. Rinse the blot five times for 5 min with TBST.
4. Incubate in the HRP conjugated secondary antibody solution for 1 hr at room temperature.

   Note: The antibody can be diluted in 3% BSA or 5% skim milk in TBST at the manufacturer's recommended ratio.
5. Rinse the blot five times for 5 min with TBST.

4 Imaging and analysis using the ChemiDoc MP system and Image Lab software (~10 min)

1. After antibody incubation, place the blot back on the sample stage of the ChemiDoc MP imager and capture a stain-free image of the blot (Image C) with the following settings:
   - Application: Stain-Free blot
   - Imaging area: Bio-Rad Criterion gel
   - Image exposure time: automatically optimized for the most intense bands

   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™ western ECL substrate mixture.
3. Mix the Clarity 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 imager.
6. Start Image Lab software and capture the chemiluminescent signals on the blot (Image D) with the following settings:
   - Application: Chemi Hi Resolution
   - Imaging area: Bio-Rad Criterion gel
   - Image exposure time: automatically optimized for the most intense bands

   Note: The image area for the stain-free and chemiluminescent images must be the same for follow-up total protein normalization by the Image Lab software.

Validate total protein normalization (~5 min)

1. Open the chemiluminescent blot image of the protein of interest and the stain-free blot image captured in the imaging step.
2. Overlay the chemiluminescent and stain-free images for normalization analysis:
   - Select the image of the protein of interest by clicking on anywhere on the image
   - Select **Normalization** from Analysis Tool Box
   - Select **Add Channel**. The image with the protein of interest populates the Sample Data field
   - Add the stain-free image by double-clicking the file name to populate the Normalization Data field

     Note: The two images may not be perfectly aligned.
   - Click **OK**
3. A window will open prompting whether to detect lanes and bands automatically on both images. Click OK, using default settings unless parameters are known.
4. Select and use **Lanes and Bands tools** to make adjustments to the lanes and bands if necessary.
5. Select **MW Analysis Tools** and assign the MW standard lanes by checking the boxes below them.
6. To view the normalized target protein volumes, click the Analysis Table on the toolbar. All calculations will be performed automatically by the software, including the Normalization Factor and Normalized Volumes. The target protein band intensity values are now adjusted for variation in the protein load. This will allow for accurate comparison of target protein among the samples.

Procedure for Multiplexing Fluorescent Western Blotting

Protocol

Please follow the steps described above for sample prep, electrophoresis, protein transfer, antibody incubation, and transfer verification. The difference between the chemiluminescent and multiplexing fluorescent western blotting procedures begins at the antibody incubation step.

3 Antibody Incubation

1. Block in 3% BSA in TBST at room temperature for 1 hr.
2. Incubate overnight at 4°C in an antibody solution containing a mouse primary antibody against the target protein 1 and a rabbit antibody against target protein 2.

   Note: The antibody should be diluted in the blocking buffer according to the manufacturer's recommended ratio. The primary antibody may be applied to the blot for 1–3 hr at room temperature depending on the antibody quality and performance.
3. Rinse the blot five times for 5 min with TBST.
4. Incubate for 1 hr at room temp in the secondary antibody solution containing a Dylight 650 conjugated goat anti-mouse antibody and a Dylight 549 conjugated goat anti-rabbit antibody.

   Note: The antibodies are diluted in 3% BSA or 5% skim milk in TBST according to the manufacturer 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.
5. Rinse the blot five times for 5 min with TBST.

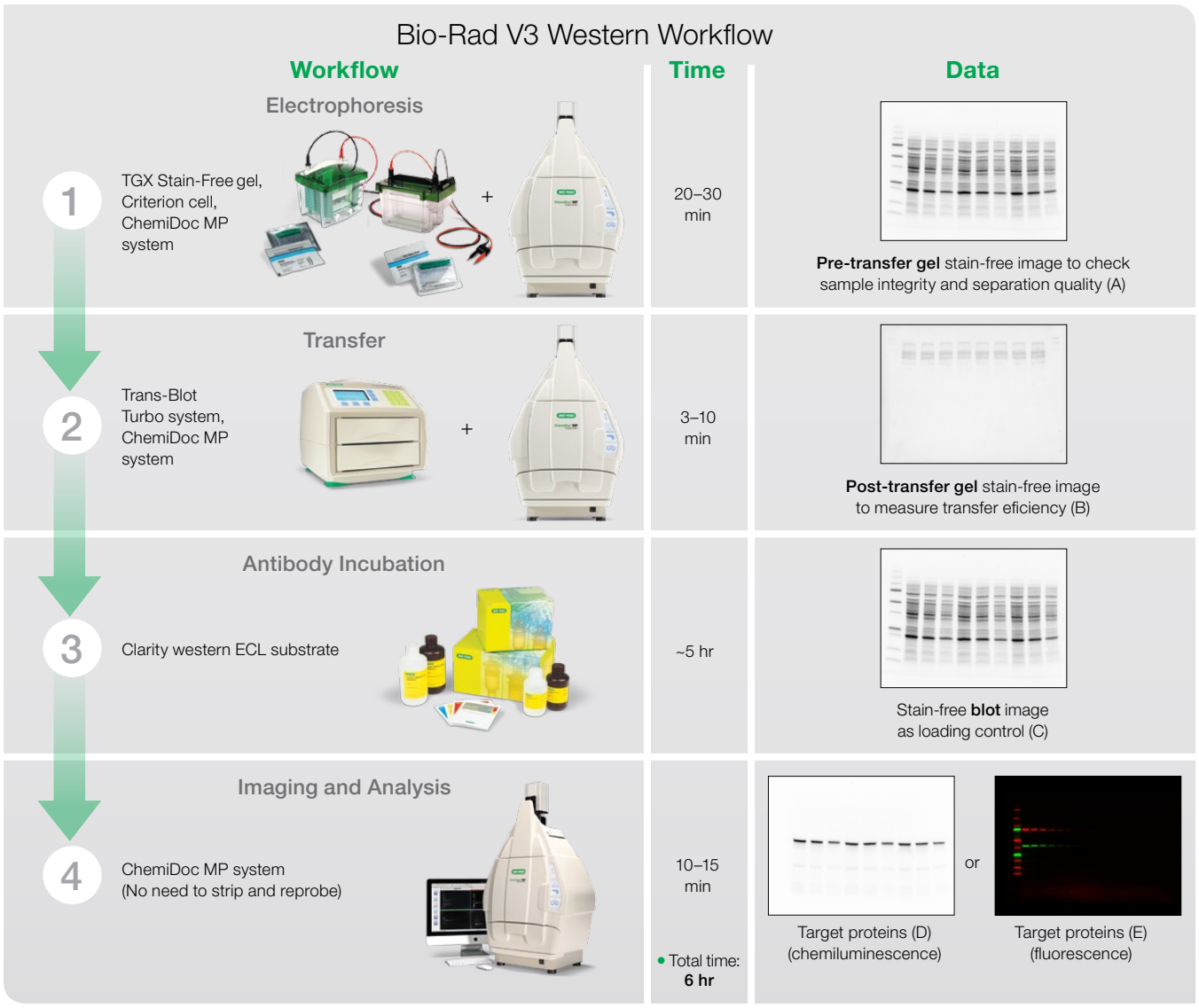
4 Imaging and analysis by Image Lab software — total protein normalization (~5 min)

1. Acquire a multiplexing fluorescent image of the blot (Image E) by opening a new multichannel protocol. Configure three fluorescent channels and run the protocol.

   Channel 1:
   - Application: blot Dylight 650
   - Imaging area: Bio-Rad Criterion gel
   - Image exposure time: automatically optimized for the most intense bands

   Channel 2:
   - Application: blot Dylight 549
   - Imaging area: Bio-Rad Criterion gel
   - Image exposure time: automatically optimized for the most intense bands

   Channel 3:
   - Application: Stain-Free blot
   - Imaging area: Bio-Rad Criterion gel
   - Image exposure time: automatically optimized for the most intense bands
2. Click the Normalization icon from the Analysis Tool Box and click Yes to detect lanes and bands.
3. Select and use **Lanes and Bands tools** to make adjustments to the lanes and bands if necessary.
4. Select **Stain-Free image** as the normalization channel.
5. Select **MW Analysis Tools** and assign the MW standard lanes by checking the boxes below them.
6. To view the normalized volumes, click the Analysis Table on the toolbar. All calculations will be performed automatically by the software, including the Normalization Factor and Normalized Volumes. The target protein band intensity values will be adjusted for variation in the protein load. This will allow for accurate comparison of target proteins among the samples.



Bio-Rad V3 Western Workflow.

The V3 Western 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 using the V3 Western Workflow, 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 V3 Western Workflow. The use of each piece of data is described. The stain-free images of the pre-transfer gel, post-transfer gel, and the 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 way that improve control and reproducibility of western blot workflow.

The target protein signals can be captured either on a chemiluminescent blot image if an HRP-conjugated secondary antibody was applied in detection (D) 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, an ɪ E were generated in a multiplex fluorescent western blotting experimenɪ. In this experiment, GAPDH was probed in 20 µg of HeLa cell lysate using a rabbit antibody (Cell Signaling Technology, Danvers, MA, 1:2500) and a Dylight 549 conjugated goat anti-rabbit antibody (Rockland, Gilbertsville, PA, 1:20,000). Protein MCM-7 was probed using a mouse antibody (Abcam, Cambridge, MA, 1:1000) and a Dylight 649 conjugated goat anti-mouse antibody (Rockland, 1:10,000). 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 (e.g. 4–15%) should be used for better transfer efficiency if the target protein size is over 100 kD.

Image D was generated from a different experiment where ß-tubulin was probed in HeLa cell lysate at different load ranging from 10–50 µg, using a mouse monoclonal ß-tubulin antibody (Rockland, 1:4,000) and an HRP-conjugated goat anti-mouse antibody (Jackson ImmunoResearch Laboratories, West Grove, PA, 1:50,000).

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