Chapter 25

Western Blotting of the Endocannabinoid System

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Abstract

Measuring expression levels of G protein-coupled receptors (GPCRs) is an important step for understanding the distribution, function, and regulation of these receptors. A common approach for detecting proteins from complex biological systems is Western blotting. In this chapter, we describe a general approach to Western blotting protein components of the endocannabinoid system using sodium dodecyl sulfate-polyacrylamide gel electrophoresis and nitrocellulose membranes, with a focus on detecting type 1 cannabinoid (CB_1) receptors. When this technique is carefully used, specifically with validation of the primary antibodies, it can provide quantitative information on protein expression levels. Additional information can also be inferred from Western blotting such as potential posttranslational modifications that can be further evaluated by specific analytical techniques.

Key words Electrophoresis, Antibody, Electrophoretic transfer, Fluorescent detection

1 Introduction

Western blotting facilitates the quantification of protein expression in a variety of tissues and allows inferences about pre- and post-translational processing (e.g., phosphorylation or alternative splicing). While Western blotting is a mature analytical technique, care must be applied in order to avoid the many pitfalls that accompany this procedure. In this chapter, we briefly describe the basic steps in Western blotting and some of the approaches we have adopted to improve efficiency, minimize artifacts, and increase the reliability of our results when detecting components of the endocannabinoid system.

2 Materials

Use distilled, deionized water for all solutions. Use analytical grade reagents whenever practical.

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2.1 Buffers and Solutions

1. Homogenization buffer: 25 mM Hepes (pH 7.4), 1 mM EDTA, 6 mM $MgCl_2$, 1 mM DTT, 1 μg/ml leupeptin, 1 μg/ml pepstatin A, and 1 μg/ml aprotinin.
2. 4× Sample buffer: 200 mM 1 M Tris–HCl (pH 6.8), 8 % sodium dodecyl sulfate, 40 % glycerol, 50 mM EDTA, 0.04 % bromophenol blue. This can be stored at room temperature for several weeks. Add 5 % (50 μl/ml) of 14.7 M β-mercaptoethanol just before use (*see* **Note 1**).
3. 10× TBS: 25 mM Tris base, 150 mM NaCl, 2.7 mM KCl. (pH 7.4). Add approximately 750 ml of water to a beaker with a large stir bar. Weigh out 30.3 g Tris base, 87.7 g NaCl, and 2 g KCl, and slowly add to stirring water. Bring pH to 7.4 with concentrated HCl and bring to 1 l volume. Autoclave and store at room temperature.
4. 10× Running buffer: 250 mM Tris–HCl (Trizma Base), 1.9 M glycine, 35 mM SDS. For 1 l of 10× running buffer: 30.25 g Tris base, 142.5 g glycine, and 10 g SDS. Mix and bring up to 1 l with ultrapure water.
5. 1× Running buffer: 100 ml of 10× running buffer + ultrapure water to a final volume of 1 l.
6. 1/2× Towbin solution: 12.5 mM Tris, 96 mM glycine, 20 % (v/v) methanol. For 1 l of Towbin buffer: 1.52 g Tris, 7.2 g glycine, and 500 ml of H_2O. Mix until salts are dissolved, add 200 ml of methanol, mix again, and bring to 1 l with H_2O.
7. Tris-buffered saline, Tween-20 (TBST): Add sufficient distilled water to 100 ml of 10× TBS to bring to 1 l. Mix in 500 μl Tween 20 until it is completely dissolved. Can store at room temperature. Should be used within several days.
8. LI-COR Biosciences Odyssey Blocking Solution.

2.2 Gel Apparatus Assembly

This protocol is based on the BioRad Mini-Protean II setup (*see* **Note 2**).

1. Materials: Short and tall glass plates (1 of each/gel), spacers (2/gel), gel-casting stand (one for every 2 gels), clamp assembly (1/gel), combs (1/gel), buffer chamber with bucket and electrode lid, U-shaped gasket, ultrapure water, and resolving and stacking gel solutions. Comb and spacers need to be of the same thickness (e.g., 0.5, 0.75, or 1.5 mm).
2. Thoroughly wash and dry glass plates with water, and then with 70 % ethanol.
3. Set up the clamp assembly and plates according to the manufacturer's instructions, making certain you tighten screws in a diagonal fashion to prevent glass from breaking.
4. Make sure that the bottom of the glass plates is aligned with the clamp assembly: misalignment will cause leakage.

5. Set clamp assembly on casting stand. Orientation of the clamp assembly is such that screws face the plastic flaps on the casting stand.
6. Make up solutions for resolving and stacking gels and degas (*see* **Note 3**); add APS and TEMED to the solution only when you are ready to pour each gel.
7. Pour the resolving gel—ensure that the height of the gel is at least 5 mm below the bottom of the plastic well-combs. Carefully (to avoid mixing), add ultrapure water or water-saturated isobutanol up to the top of the short plate and let resolving gel polymerize (≥30 min).
8. Decant the water and pour the stacking gel. Carefully add the well-combs to avoid bubbles and let stacking gel polymerize (30 min).
9. Once the stacking gel has polymerized, gently remove the comb and carefully rinse the wells with 1× running buffer to remove bubbles and unpolymerized acrylamide.

Recipes for 7.5 and 10 % Tris/glycine gels for the BioRad Miniprotean system. Resolving gels of other concentrations can be produced by altering the ratios of 30 % acrylamide and water as necessary.

Tris/glycine				
Resolving	**10 % (1 thick)**	**10 % (2 thick)**	**7.5 % (1 thick)**	**7.5 % (2 thick)**
30 % Acryl	3.3 ml	6.6 ml	2.5 ml	5.0 ml
1.5 M Tris pH 8.8	2.5 ml	5 ml	2.5 ml	5.0 ml
H_2O	3.95 ml	7.9 ml	4.9 ml	9.8 ml
20 % SDS	50 µl	100 µl	50 µl	100 µl
10 % APS	80 µl	160 µl	100 µl	200 µl
TEMED	8 µl	16 µl	8 µl	16 µl
Stack	1 thick	2 thick	4 thick	
30 % Acryl	665 µl	1330 µl	2660 µl	
1.0 M Tris pH 6.8	625 µl	1250 µl	2500 µl	
H_2O	1.5 ml	3 ml	6 ml	
50 % glycerol	1.5 ml	3 ml	6 ml	
20 % SDS	25 µl	50 µl	100 µl	
10 % APS	20 µl	40 µl	80 µl	
TEMED	5 µl	10 µl	20 µl	
1.5 M Tris base, pH 8.8	18.17 g/100 ml			
1.0 M Tris base, pH 6.8	12.11 g/100 ml			

2.3 Gel Electrophoresis and Blotting Equipment

1. There are many gel blotting systems available that provide excellent transfer of protein from gel to support membranes such as nitrocellulose. We use a 15 × 17 cm transfer unit from Idea Scientific Company.
2. Transfer: Transfer unit complete with sponges and electrodes, square-Pyrex pan, 1/2×-Towbin solution, filter paper (2/gel), nitrocellulose membrane (1/gel).
3. Fluorescent scanner capable of dual-infrared wavelength detection (e.g., LiCor Odyssey).

3 Methods

A variety of samples can be used as starting material for Western blotting. Primarily we use tissue homogenates and cultured cell lysates in our studies. Generally, it is best to process samples from live organism/cell until separation on the gel in a single day to minimize proteolytic loss and protein aggregation. At all times, keep samples cold (e.g., on ice). If necessary, lysates can be frozen (preferably at −80 °C) in sample buffer. They should be rapidly thawed, and spun at 3000 × *g* for 10 min, and pellet discarded before separation on the gel.

3.1 Typical Tissue Preparation

1. Place 3 ml of homogenization buffer into Falcon 2059 tubes, weigh, and place on ice.
2. Sacrifice animals and transfer dissected tissue to pre-chilled tubes and immediately homogenize with Polytron homogenizer.
3. Re-weigh tubes and adjust volume of buffer to 10:1 (vol:wt). Mix gently.
4. Spin samples at 700 × *g* and 4 °C for 5 min. Transfer supernatants to sterile tubes and resuspend pellets with original 10:1 volume of buffer. Repeat homogenization and spin samples at 700 × *g* for 5 min and 4 °C.
5. Pool like supernatants and centrifuge at 16,000 *g* for 30 min and 4 °C.
6. Discard supernatants and resuspend pellets in original 10:1 volume of homogenization buffer. Triturate thoroughly to wash membranes. Centrifuge samples again at 16,000 × *g* for 30 min and 4 °C.
7. Aspirate supernatants and resuspend pellets in suitably small volumes of homogenization buffer. Quantitate protein concentrations via Bradford Assay.
8. Mix desired amount of protein with 1:3 volume of 4× sample buffer, heat for 5 min at 65 °C (be aware that *boiling causes CB1 to aggregate and the aggregated receptor separates poorly on the resolving gel*), and load onto a gel.

3.2 Preparing Cultured Cells

1. Grow cells of interest on appropriate-size culture plates. In this example, we use 6-well plates. Treat cells as required for the experiment.
2. Place plate on ice, aspirate medium, and add 250 µl of homogenization buffer containing 0.5 % CHAPS. Scrape cells and homogenize in a dounce, or polytron homogenizer. Transfer lysate to 1.5 ml microfuge tubes and spin down samples at 700 × *g* and 4 °C for 10 min. Collect supernatant and add an equal volume of 2× SDS loading buffer.
3. Incubate tubes in 65 °C water bath for 5 min (you may need to sonicate proteins prior to water bath incubation, particularly for samples that are rich in nucleic acids, which may gel and impede efficient and quantitative sample handling).
4. Spin at 100 × *g* for 2 min, transfer supernatant to a fresh Eppendorf tube, and load onto a gel.

3.3 Running the Gel

1. Load samples and molecular marker using volumes appropriate for well size and gel thickness (*see* **Note 4**).
2. Using an L-shaped glass pipette, dislodge the bubbles at the bottom of the clamp assembly with a gentle stream of 1× running buffer. This ensures even current distribution throughout the gel.
3. Run the gel at 80 V until you see higher molecular weight marker bands of the ladder begin to separate (*see* **Note 5**).
4. Increase the voltage to ~120 V and let the gel run until the blue dye front runs off (1–1.5 h for minigels).
5. Electrophoresis unit can be placed in cold room or refrigerated cabinet when running at higher voltages to minimize heat artifacts (*see* **Note 6**).

3.4 Transferring the Gel

Soak nitrocellulose membrane first in water, and then in 1/2× Towbin buffer to hydrate. Also, immerse transfer sponges and filter paper in 1/2× Towbin buffer prior to setting up the transfer.

1. Disassemble the gel unit by loosening screws on clamp assembly and gently removing the top plate and spacers to expose the gel.
2. Remove the stacking gel from the resolving gel using the long edge of a spacer.
3. Set up the transfer so the gel and nitrocellulose are sandwiched between two pieces of filter paper, which is sandwiched between two transfer sponges. The current will run from the cathode to anode plate, so the gel and nitrocellulose should be situated so negatively charged protein from the gel runs onto the nitrocellulose. Be careful to remove all air pockets between the nitrocellulose and gel (*see* **Note 7**).

4. After setting up the upright transfer unit, check the level of buffer solution. The gel and filter paper must be completely submerged in buffer during transfer.
5. Run the transfer at 30–40 V for 1 h (voltage and time depend on transfer unit used; see manufacturers' instruction for more information).

3.5 Western Blot

1. Briefly soak nitrocellulose in TBS. (*Optional: Check success of the protein transfer by soaking nitrocellulose in Ponceau-S for 15 min. Wash Ponceau-S off with reagent-grade water. Do not use Tween in wash at this point, as this will significantly increase background on Odyssey scanner.*) (*See* **Note 8**.)
2. Incubate nitrocellulose in Licor blocking solution for 1 h at room temperature.
3. Incubate nitrocellulose overnight at 4 °C in primary antibody solution (primary antibody in PBS + 1:1 (Licor block:1× PBS). Dilution of primary antibody needs to be empirically determined (*see* **Note 9**).
4. The following day, wash nitrocellulose in TBS-T with four 15-min washes.
5. Incubate nitrocellulose in secondary antibody solution (secondary antibody (1:5000) + 1:1 Licor block:1× PBS) for 1 h at room temperature.
6. Repeat the four 15-min TBS-T washes.
7. Wrap blot in Saran wrap and scan with Odyssey.

4 Notes

1. SDS binds to the protein in an amount that is typically (but not always) proportional to the protein's molecular weight, allowing proteins to migrate according to size. Glycerol increases sample density relative to the buffer, facilitating efficient loading. Beta-mercaptoethanol is a strong reducing agent, which lessens protein aggregation via disulfide bond formation.
2. Pre-poured gels can be used in systems such as the Bio-Rad Mini-Protean and Thermo Fisher Scientific XCell SureLock systems. Their use will simplify setup.
3. We use house vacuum for 10–15 min to remove excess dissolved gas from the gel solution. Polymerization requires the formation of SO_4^-. Because oxygen serves as a free radical trap, excess amounts of this gas in the gel solution can result in non-uniform polymerization of the gel.
4. The loading capacity of a well is determined by the size of the comb's teeth. For example, teeth with dimensions of

1 mm × 6 mm × 8 mm will have a capacity of 48 μl. However, we have found that banding will be tighter and more uniform using smaller volumes/well. Of course, loading volume is determined by the protein concentration of the sample. It is therefore important not to over-dilute your lysates while processing. It is also desirable that the ionic composition of samples on a single gel be similar. Marked differences in salt concentrations will result in spreading and narrowing of bands.

5. Protein ladders such as SeaBlue plus 2 from Invitrogen have dye-conjugated protein bands. These can be used to determine when proteins of a certain size have passed the interface. For instance, the SeaBlue plus 2 has an orange phosphorylase band that migrates at 148 kDa. Voltage can be increased once higher molecular weight bands have clearly separated.
6. Voltage should be kept low (5–10 V/cm) until protein bands have been compressed at the interface between stacking and resolving gels. Once higher molecular weight marker bands have separated, the voltage can be increased to 10–15 V/cm. Too high a voltage can result in overheating and a breakdown of the gel matrix. This can appear as diffuse banding or uneven migration of bands. This can be partially overcome by chilling buffers with a cooling block and/or running gels in a cold room.
7. With the gel sandwich just submerged in Towbin's buffer, roll a smooth tube over the top piece of chromatography paper with gentle pressure. Work from the center of the paper to one end, and then from the center to the other end. Do this several times to make sure that all bubbles are squeezed out.
8. Proteins on blots can be quickly quantified using Ponceau S staining. After protein transfer, the blot is briefly rinsed in TBS, and then soaked in 0.1 % (w/v) Ponceau S in 5 % acetic acid for 15 min at room temperature with gentle rocking. The blot is then rinsed several times in reagent-grade water until bands can be clearly seen. With this technique, the blot can be photographed or scanned as shown in Fig. 1. This staining can be used to normalize protein loading rather than using a housekeeping antibody, such as β-actin (Fig. 1). Using Ponceau S in this way opens up the second channel on a dual-wavelength scanner for analyzing expression levels of a second protein of interest.
9. The primary antibody is the most important element of Western blotting. A good antibody will often show strong specific bands even under suboptimal conditions, while a poor antibody will show strong nonspecific bands no matter how the procedure is optimized. Signal can be improved by adjusting protein loading and/or antibody concentrations. Antibody specificity should be determined by running negative control lysates lacking the antigen of interest. Examples are mock-

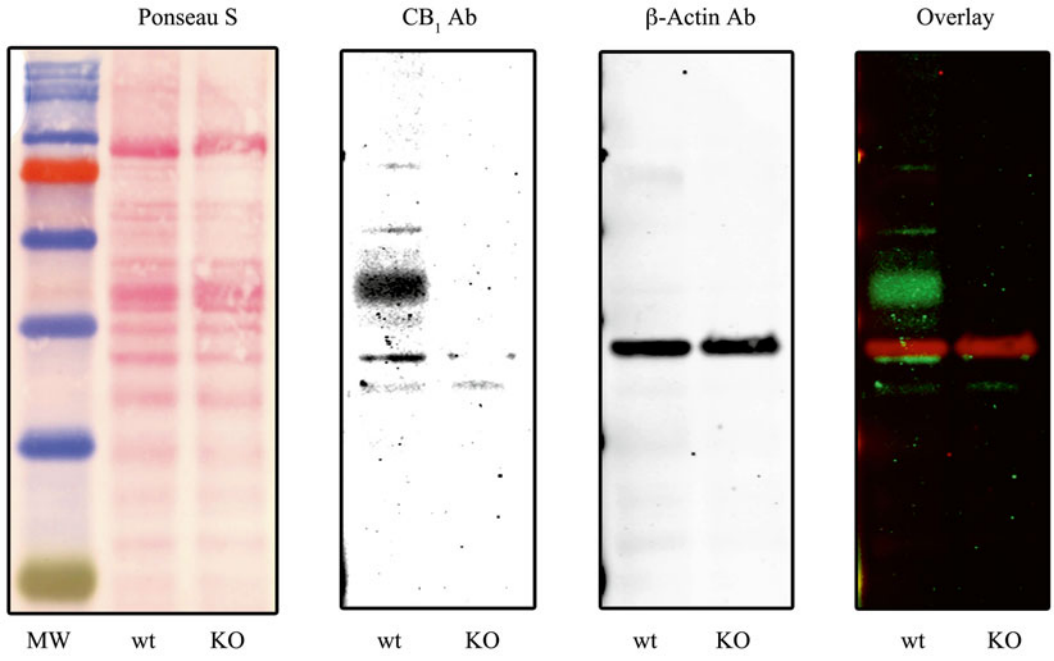

Fig. 1 Western blot showing CB_1 staining in wild-type versus CB_1 knockout mouse brain lysates. Wells were loaded with 25 μg protein (cortical homogenate). The first panel shows Ponceau-S staining of blot prior to antibody incubation. The second and third panels show grayscale images of 800 nm and 700 nm channels, respectively. The last panel shows an overlay of CB_1 staining (*green*) and actin staining (*red*). Primary antibodies used were rabbit anti-CB_1-carboxy terminus antibody (diluted 1:500), made in our laboratory, and mouse anti-actin IgM (diluted 1:5000) from Developmental Studies Hybridoma Bank (http://dshb.biology.uiowa.edu/actin). Secondary antibodies used were goat anti-mouse IgM IR_{680}RD (LI-COR Biosciences, www.licor.com), and Donkey anti-rabbit IgG IR_{800} (Rockland Inc. www.rockland-inc.com). Both secondary antibodies were diluted 1:5000

transfected cell lines and tissue lysates from knockout animals. An alternative approach is to selectively alter the mobility of the protein of interest, such as by deglycosylation. While peptide blocking is often used to show antibody specificity, this approach can lead to spurious results since many proteins share common epitopes. It is also important to note that antibody specificity needs to be demonstrated for each application. An antibody that is specific for Western blotting may not be specific for immunocytochemistry and vice versa. As discussed above, the best controls are lysates that are identical (e.g., processed at the same time and in the same fashion) to the test samples saved for the antigen of interest.