

# Chapter 2

## Impact of Blocking and Detection Chemistries on Antibody Performance for Reverse Phase Protein Arrays

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### Abstract

Careful selection of well-qualified antibodies is critical for accurate data collection from reverse phase protein arrays (RPPA). The most common way to qualify antibodies for RPPA analysis is by Western blotting because the detection mechanism is based on the same immunodetection principles. Western blots of tissue or cell lysates that result in single bands and low cross-reactivity indicate appropriate antibodies for RPPA detection. Western blot conditions used to validate antibodies for RPPA experiments, including blocking and detection reagents, have significant effects on aspects of antibody performance such as cross-reactivity against other proteins in the sample. We have found that there can be a dramatic impact on antibody behavior with changes in blocking reagent and detection method, and offer an alternative method that allows detection reagents and conditions to be held constant in both antibody validation and RPPA experiments.

**Key words:** Reverse phase protein array, Blocking buffer, Antibody validation, Detection chemistry, Near-infrared fluorescence

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### 1. Introduction

Reverse phase protein array (RPPA) analysis is a high-throughput technique that has been used to characterize cancer signaling pathways (1–4) and identify characteristic changes which may define a set of diagnostic and prognostic biomarkers (5). Lysates of whole cells, microdissected tissues, or other patient samples are applied to nitrocellulose-coated glass slides followed by probing with one or two analyte-specific antibodies that can be detected by colorimetric, chemiluminescent, amplified fluorescent, or near infrared methods (6, 7). The most critical aspect of RPPA success is validation and selection of appropriate antibodies for detection.

Antibodies must be highly specific as demonstrated by a single band on a Western blot (8). Western blot chemistries consist of not only antibodies, but also blocking agent for decreasing background, and several different signal generating approaches. Since RPPA detection is based on the same immunodetection principles as Western blot detection, such changes can also significantly affect antibody reactivity in RPPA and therefore, impact the quantification and analysis of the experiment. The use of blocking conditions for RPPA analysis that differs from those used for the initial Western blot antibody validation has been shown to significantly alter the data obtained from the RPPA experiment (9, 10).

Near infrared detection for antibody validation using Western blots and RPPA slides offers a sensitive, quantitative, and accurate way to identify protein changes in RPPA. The method below is designed to optimize antibody performance by testing multiple blocking buffers. The RPPA is then detected using the optimized blocking buffer with the exact same antibodies and near infrared detection procedure, thereby eliminating any bias caused by changing detection chemistries.

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## 2. Materials

### **2.1. SDS-Polyacrylamide Gel Electrophoresis for Blocker Optimization**

1. Tissue Lysate: Mouse and rat thymus, liver, and brain tissue available from BIOMOL International L. P. (Plymouth Meeting, PA). Store at  $-80^{\circ}\text{C}$  (see Note 1).
2. 4–20% Tris–Glycine Novex™ Gel, 15-well (Invitrogen, Carlsbad, CA). Store at  $4^{\circ}\text{C}$  (see Note 2).
3. Running buffer: 25 mM Tris, 192 mM glycine. Store at room temperature.
4. Protein loading buffer (2×): 62.5 mM Tris–HCl, pH 6.80, 25% (v/v) glycerol, 2% (v/v) SDS, 1% (w/v) Orange G, 5% (v/v)  $\beta$ -mercaptoethanol. Store at room temperature.
5. Prestained molecular weight markers: Two-Color Protein Markers (LI-COR, Lincoln, NE). Store in aliquots at  $-20^{\circ}\text{C}$ .

### **2.2. Western Blotting for Blocker Optimization**

1. Odyssey® nitrocellulose membrane from LI-COR and 3 MM Chr chromatography paper from Whatman, Maidstone, UK (see Note 3).
2. Transfer buffer: 25 mM Tris, 192 mM glycine, 0.1% SDS, 20% (v/v) methanol. Store at  $-20^{\circ}\text{C}$ .
3. 1× Phosphate-buffered saline (PBS): 137 mM NaCl, 2.7 mM KCl, 4.3 mM  $\text{Na}_2\text{HPO}_4$ , 1.47 mM  $\text{KH}_2\text{PO}_4$ , pH 7.4. Store at room temperature (see Note 4).

4. 1× PBS Tween®-20 (PBST): 137 mM NaCl, 2.7 mM KCl, 4.3 mM Na<sub>2</sub>HPO<sub>4</sub>, 1.47 mM KH<sub>2</sub>PO<sub>4</sub>, pH 7.4, 0.1% (v/v) Tween-20. Store at room temperature.
5. Blocking buffers: Odyssey blocking buffer (LI-COR, Lincoln, NE), 5% (w/v) nonfat dry milk in PBS, 5% (w/v) bovine serum albumin (BSA) in PBS. Store at 4°C (see Note 5).
6. Primary and secondary antibody diluents: Odyssey blocking buffer, 0.2% (v/v) Tween-20; 5% (w/v) nonfat dry milk in PBS, 0.2% (v/v) Tween-20; 5% (w/v) BSA in PBS, 0.2% (v/v) Tween-20. Store at 4°C.
7. Primary antibody: Rabbit anti-ERK 1 (K-23) (Santa Cruz Biotechnology, Inc., Santa Cruz, CA). Store at 4°C (see Note 6).
8. Secondary antibody: IRDye® 800CW Goat anti-rabbit (LI-COR). Store at 4°C (see Note 7).

**2.3. Reverse Phase  
Protein Array  
Detection Using  
Optimized Detection  
Chemistry**

1. Panorama™ Mouse/Rat Tissue Extract Protein Array (Sigma, St. Louis, MO). Store at room temperature (see Note 8).
2. 1× PBS: 137 mM NaCl, 2.7 mM KCl, 4.3 mM Na<sub>2</sub>HPO<sub>4</sub>, 1.47 mM KH<sub>2</sub>PO<sub>4</sub>, pH 7.4. Store at room temperature (see Note 4).
3. 1× PBS Tween-20 (PBST): 137 mM NaCl, 2.7 mM KCl, 4.3 mM Na<sub>2</sub>HPO<sub>4</sub>, 1.47 mM KH<sub>2</sub>PO<sub>4</sub>, pH 7.4, 0.1% (v/v) Tween-20. Store at room temperature.
4. Blocking buffers: Odyssey blocking buffer (LI-COR), 5% (w/v) nonfat dry milk in PBS, 5% (w/v) BSA in PBS. Store at 4°C (see Note 9).
5. Primary and secondary antibody diluents: Odyssey blocking buffer, 0.2% (v/v) Tween-20; 5% (w/v) nonfat dry milk in PBS, 0.2% (v/v) Tween-20; 5% (w/v) BSA in PBS, 0.2% (v/v) Tween-20. Store at 4°C (see Note 10).
6. Primary antibody: Rabbit anti-ERK 1 (K-23) (Santa Cruz Biotechnology, Inc., Santa Cruz, CA). Store at 4°C (see Note 6).
7. Secondary antibody: IRDye 800CW Goat anti-rabbit (LI-COR). Store at 4°C (see Note 7).

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## **3. Methods**

### **3.1. SDS-PAGE for Blocker Optimization**

1. Prepare samples by placing 20 µL (100 µg) each of mouse brain, rat brain, mouse liver, rat liver, mouse thymus, and rat thymus tissue extracts into different 0.5 mL microcentrifuge tubes and label with contents (see Note 11). Add 20 µL of 2× protein loading buffer to each extract sample. Mix by gently pipetting up and down. Cap tubes and place at 100°C for 5 min.

Remove from heat and place directly on ice until ready to load gel. Centrifuge briefly to collect sample to bottom of tube.

2. These instructions utilize the XCell SureLock™ Mini-Cell Electrophoresis Apparatus (Invitrogen) for electrophoresis. Remove tape strip from bottom of two 4–20% Tris–glycine Novex gels. Assemble according to XCell SureLock Mini-Cell Instruction Manual. Remove gel combs. Fill box with 1× running buffer. Using pipette gently rinse out the wells with buffer in buffer tank.
3. For two gels, load lanes 1, 8, and 15 with 5 µL of Two-Color Protein Markers. In lanes 2–7, place 10 µL tissue extract samples in above order and repeat in lanes 9–14.
4. Fully assemble XCell SureLock Mini-Cell Electrophoresis Apparatus and plug into power supply. Run the gel at a fixed voltage of 125 V for 100 min.

### **3.2. Western Blotting for Blocker Optimization**

1. Following electrophoresis, transfer the samples to supported nitrocellulose membrane. It is important that the membrane only be handled by the edges with clean forceps. Take great care to never touch the membrane with bare or gloved hands (see Note 12).
2. This procedure describes the use of Bio-Rad Mini Trans-Blot® Electrophoretic Transfer Cell tank system (see Note 13). While gel is running, fill Bio-Ice cooling unit with distilled water and place in –20°C. Cut two pieces of the nitrocellulose membrane to 7×8 cm size with a paper cutter designated for membrane cutting (i.e., does not get used for general purpose). Place two pieces of cut nitrocellulose membrane into a Rubbermaid container (710 mL rectangle). Place four pieces of Whatman paper 3 MM, 7×8 cm into the same plastic container (710 mL rectangle). Cover the nitrocellulose and Whatman paper with transfer buffer. Place four fiber pads into a different plastic container (710 mL rectangle). Cover the fiber pads with transfer buffer. Let fiber pads, nitrocellulose, and Whatman paper soak while gel is running.
3. After electrophoresis open the gel cassette using the gel tension wedge. Trim the bottom of the gel off just above the loading dye. Pour 200 mL of 1× transfer buffer into a plastic container (710 mL rectangle). Carefully place gels in 1× transfer buffer. Gently shake on platform shaker for 5 min.
4. Prepare gel sandwich and assemble transfer cell according to the Mini Trans-Blot Electrophoretic Transfer Cell Instruction Manual, Bio-Rad. Plug into power supply and run at a fixed voltage of 100 V for 65 min.
5. Disassemble transfer cells and remove blots from transfer unit. Place in between two sheets of Whatman paper. Let air dry

overnight. Blots can be stored dry at 4°C for up to 3 months before being processed.

6. Cut both membranes down the middle of the protein marker in lane 8, being careful not to touch the membrane. Label with pencil appropriately.
7. Place membranes into three different Western blot incubation boxes. There will be one extra membrane that can be used as a backup or for a fourth blocking condition. In box 1 add 10 mL of Odyssey blocking buffer; in box 2 add 10 mL of 5% (w/v) nonfat dry milk in PBS; in box 3 add 5% (w/v) BSA in PBS and block the membranes for 1 h with gentle shaking.
8. Dilute ERK 1 primary antibody 1:1,000 in 10 mL of diluent as follows: For box 1, dilute in Odyssey blocking buffer diluent; for box 2, dilute in 5% (w/v) nonfat dry milk diluent; for box 3, dilute in 5% (w/v) BSA diluent (see Note 6).
9. For all blots, decant off blocking buffer and add the diluted ERK antibody. Incubate blots overnight at 4°C with gentle shaking (see Note 14).
10. Decant off primary antibody solution. Rinse membrane with 1× PBST. Cover blot with 10 mL of 1× PBST. Shake vigorously on platform shaker at room temperature for 5 min. Decant off wash solution. Repeat three additional times.
11. Dilute IRDye® 800CW Goat anti-rabbit antibody 1:10,000 in 10 mL of diluent as follows: For box 1, dilute in Odyssey blocking buffer diluent; for box 2, dilute in 5% (w/v) nonfat dry milk diluent; for box 3, dilute in 5% (w/v) BSA diluent (see Note 7).
12. Add the diluted secondary antibody to the appropriate boxes. Incubate blots for 1 h at room temperature with gentle shaking. Protect membranes from light during incubation by covering with foil or a cardboard box.
13. Decant off secondary antibody solution. Rinse membrane with 1× PBST. Continue to protect the membranes from light during washes. Cover blot with 10 mL of 1× PBST. Shake vigorously on platform shaker at room temperature for 5 min. Decant off wash solution. Repeat three additional times.
14. Rinse membranes with 1× PBS to remove residual Tween-20. The membranes are now ready to be imaged.
15. Scan wet blots on Odyssey Infrared Imaging System by placing them face down on the glass surface and image with the following settings: resolution = 169  $\mu$ m, quality = medium, focus offset = 0.0 mm, intensity = 5(800) (see Note 15).
16. Select a “blocking buffer” condition from the three that gives the least amount of nonspecific banding and the highest signal intensity to move forward with the RPPA processing (see Note 16). For this target, Odyssey blocking buffer is the buffer of choice. An example of the results produced is shown in Fig. 1.

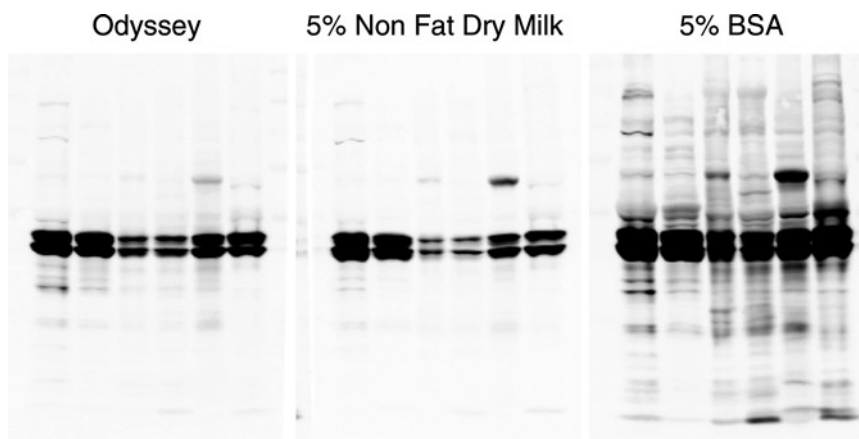


Fig. 1. ERK 1 antibody performance in Odyssey blocking buffer, 5% nonfat dry milk, and 5% BSA. Tissue lysates are as follows: *lane 1* – mouse brain, *lane 2* – rat brain, *lane 3* – mouse liver, *lane 4* – rat liver, *lane 5* – mouse thymus, *lane 6* – rat thymus. Odyssey blocking buffer was chosen for detection of the RPPA. Blots were imaged on Odyssey Infrared Imaging System at the following settings: resolution = 169  $\mu$ m, quality = medium, focus offset = 0.0 mm, intensity = 5(800).

### 3.3. Reverse Phase Protein Array Detection Using Optimized Detection Chemistry

1. Label Panorama™ Mouse/Rat Tissue Extract Protein Array with target using a pencil.
2. Place slide in a small incubation box. Incubate the slide for 10 min in pre-incubation buffer. Aspirate the pre-incubation buffer from the box. Add enough Odyssey blocking buffer to the incubation box to completely submerge the slide. Incubate with gentle rocking for 40 min at room temperature.
3. Dilute ERK 1 primary antibody 1:1,000 in 4 mL of Odyssey blocking buffer diluent (see Note 17).
4. Aspirate Odyssey blocking buffer out of the incubation box. Add diluted primary antibody to the slide. Incubate slide overnight at 4°C with gentle shaking.
5. Aspirate primary antibody solution. Cover slide with 1× PBST. Shake vigorously on platform shaker at room temperature for 5 min. Decant off wash solution. Repeat three additional times.
6. Dilute IRDye 800CW Goat anti-rabbit antibody 1:10,000 in 4 mL of Odyssey blocking buffer diluent (see Note 17).
7. Add diluted secondary antibody to the slide. Incubate slide for 1 h at room temperature with gentle shaking. Protect slide from light during incubation by covering with foil or a cardboard box.
8. Aspirate secondary antibody solution. Cover slide with 1× PBST. Continue to protect the membranes from light during washes. Shake vigorously on platform shaker at room temperature for 5 min. Pour off wash solution. Repeat three additional times. Rinse slide with 1× PBS to remove residual Tween-20.

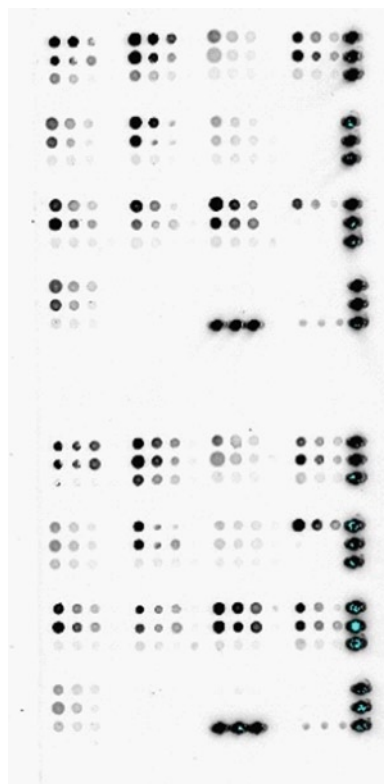


Fig. 2. ERK1 antibody performance using Odyssey blocking buffer on a Sigma Panorama mouse and rat tissue RPPA. Array was imaged on Odyssey Infrared Imaging System at the following settings: resolution=42  $\mu\text{m}$ , quality=medium, focus offset=0.0 mm, intensity=5(800).

9. Using a slide carrier, centrifuge the slide dry to eliminate as much liquid as possible. Allow slide to air dry in the dark for 30 min before imaging.
10. Scan dry slide on Odyssey Infrared Imaging System by placing it protein side down on the glass surface and image with the following settings: resolution=42  $\mu\text{m}$ , quality=medium, focus offset=0.0 mm, intensity=5(800) (see Note 15). An example of the results produced is shown in Fig. 2.

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## 4. Notes

1. Mouse and rat tissue were chosen for this example. When validating antibodies for RPPA, a representative sample from the array should be used for Western blot validation.
2. This protocol should be adapted for an SDS-polyacrylamide gel electrophoresis (SDS-PAGE) electrophoresis system that is

optimal for both protein sample as well as the size of the target protein. Buffer composition and percent acrylamide can be altered.

3. Not all nitrocellulose is optimal for use on the Odyssey Infrared Imaging System. Some nitrocellulose has more background fluorescence than others. Use caution if using another brand of nitrocellulose.
4. If the primary antibody being evaluated performs optimally in Tris-buffered saline (TBS) replace all PBS buffers with TBS.
5. Three blocking buffers were chosen for this procedure; however, it can be modified for other blocker choices. The addition of 0.2% Tween-20 to the blocking buffer is critical for the primary and secondary antibody diluents to reduce background. It is important to note that some blockers already have detergent in them and additional Tween-20 may not be optimal.
6. Any primary antibody can be substituted. Dilutions of primary antibody may need to be optimized. Vendor recommended dilutions for Western blot applications are generally the best place to begin.
7. The choice of secondary antibody will vary depending on the host species of the primary antibody being evaluated. IRDye 800CW conjugated secondary antibodies are optimal as there is very little autofluorescence of the membrane surfaces and biomolecules in the 800 nm range of the spectrum. Secondary antibody dilutions may need to be optimized. Typical dilution recommendations are 1:5,000–1:25,000.
8. Panorama Mouse/Rat tissue extract arrays were used to optimize this procedure. Any RPPA on nitrocellulose-coated glass slides can be substituted. RPPA with spot sizes greater than 200  $\mu\text{m}$  in diameter will result in the best quantification results with infrared detection on the Odyssey Infrared Imaging System.
9. The blocking buffer that is chosen from the Western blot antibody optimization should be the blocking buffer used for the RPPA.
10. The primary and secondary antibody diluents for use with RPPA will correspond to the blocking treatment that was chosen in Western blot antibody optimization.
11. This procedure utilized 25  $\mu\text{g}$  of tissue lysate in each well of the gel. Depending on the sample type and target of interest 5–25  $\mu\text{g}$  of sample may be optimal.
12. When detecting membranes in the near infrared it is important not to contaminate the membrane. The most common contaminants are blue pen, Coomassie stain, poorly cleaned incubation containers, and fingerprints.

13. This procedure can be adapted to most wet transfer units by following manufacturer's recommendations.
14. Primary antibody incubation may need to be optimized. Typical recommendations are 1–4 h at room temperature or overnight at 4°C with gentle shaking.
15. The scan intensity may need to be optimized depending on the sensitivity needs of the blot. If the image contains saturation the scan intensity will need to be reduced. Weak band signal could be improved by increasing the scan intensity. It is important to recognize that background may increase as well.
16. Quantification of the blots can be done using the Odyssey application software.
17. Volume will vary depending on the size of container used for incubations. The volume in this procedure is for use with the incubation plates that come with the Panorama arrays.

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