

Protocol for Printing Slides and Antibody Labeling

Cell or tissue lysates are arrayed on nitrocellulose-coated slides (Grace Bio-labs, Bend, OR) in triplicate using the Aushon 2470 Arrayer (Aushon BioSystems, Billerica, MA) (technical replicates). Spotted slides are stored in a sealed Ziploc bag at -20°C. Immunolabeling is performed on an automated slide stainer Autolink 48 (Dako, Carpinteria, CA) according to the manufacturer's instructions. Each slide is incubated with a single primary antibody at room temperature for 30 minutes followed by a goat anti-rabbit or anti-mouse IgG secondary antibody. A negative control slide is incubated with antibody diluent instead of primary antibody. The VECTASTAIN Elite ABC-HRP Kit (Vector Laboratories, Newark, CA) and Tyramide Signal Amplification plus biotin kit (Akoya Biosciences, Marlborough, MA) are used as part of the catalyzed signal amplification system while fluorescent IRDye 680 Streptavidin (Li-Cor Biosciences, Lincoln, NE) is used as the detection system. Slides are stained in batches with one negative control per batch; each antibody stained and scanned batch takes 1 day. Total protein for each spot is assessed by staining one in every 50 slides with Sypro Ruby Blot Stain (Molecular Probes, Eugene, OR) according to the manufacturer's directions. Slides are scanned on a GenePix AL4400 scanner (at 635 nm wavelength for antibody slides or 535 nm wavelength for Sypro Ruby Blot stained slides), and the images are analyzed by the GenePix Pro 7.2 software (Molecular Devices, Sunnyvale, CA). The fluorescence signal of each spot is obtained from the fluorescence intensity after subtraction of the local slide background signal.

RPPA data normalization details:

We select 1 slide in every 50 (in printing order) for total protein staining and use that to perform total protein normalization across all the samples. The normalization method is described below:

A group-based normalization approach is used to normalize the RPPA data. For each spot on the array, the background-subtracted foreground signal intensity (SI) is used as the spot's SI; if the background intensity is higher than the foreground intensity, the spot's SI is set to 1 (a very small intensity value). To normalize the antibody SI, the antibody SI of each spot is subtracted by the corresponding SI of the negative control and then normalized to the corresponding SI of total protein within the same group. For each spot, the normalized antibody SI can be expressed using the following formula:

$$N = \frac{A - C}{T} \times M$$

where N is the normalized antibody SI, A is the raw antibody SI, C is the negative control SI, M is the median SI of the spots of the same group, and T is the SI of total protein. In addition, for each spot, if the antibody SI is lower than the negative control SI, the normalized SI is set to 1; if the antibody SI, the negative control SI, or the total protein SI had a flag indicating the SI is problematic, the normalized antibody SI is set to non-applicable (NA).

Protocol for antibody labeling:

- 1) TBST (0.05M Tris-HCl 0.3M NaCl with 0.1% Tween-20) (5 min)
- 2) I-block (15 min x 2)
- 3) TBST
- 4) H₂O₂ (5 min)
- 5) Avidin block (10 min)
- 6) TBST
- 7) Biotin block (10 min)
- 8) TBST (2X)
- 9) Primary antibody (30 min)

- 10) TBST (5 min)
- 11) Biotinylated secondary antibody (15 min)
- 12) TBST (5 min)
- 13) SABC (Streptavidin-Biotin Complex)
- 14) TBST (5 min)
- 15) TSA reagent (15 min)
- 16) TBST (5 min)
- 17) Streptavidin-IRDye-680 (LiCor) (15 min)
- 18) ddH₂O (1 min)
- 19) Rinse twice with ddH₂O
- 20) Dry slides
- 21) Scan slides

Slide spotting format:

- a. Experimental lysates
- b. Positive, negative, and other control lysates:
 - 1) **Cell lysate mix controls:** we have selected four cell lines (breast cancer cell lines MDA-MB-415 and T47D, pervanadate-treated HeLa cells, and calyculin A-treated cells) that express the majority of the antigens to be assayed. The mixed control lysate is serially diluted over a range of 0.0078 mg/ml to 1 mg/ml to test for the linear range of antibody response.
 - 2) **NCI 60 cancer cell lines:** lysates of a mix of 60 cancer cell lines.
 - 3) **Breast Cancer ATCC 39 cell lines:** lysates of a mix of 39 breast cancer cell lines.
 - 4) **Mouse tissue mix:** cell lysates from multiple mouse tissues are spotted as positive controls for mouse proteins.
 - 5) **IgG mix:** rabbit, mouse, and goat IgG are mixed at 0.05 mg/ml and spotted at four corners of the slides as gridding and positive controls.
 - 6) **BSA for non-specific binding controls:** one or two different sources of BSA are spotted at 0.5 mg/ml as controls.
 - 7) **RPPA cell lysis buffer** as a negative control.
 - 8) **Calibrators:**
 - a. Cell lysates of **Pervanadate treated HeLa cells** mixed with untreated HeLa lysate at 0-100% dilution for tyrosine phosphorylated antibodies.
 - b. Cell lysates of **Calyculin A-treated Jurkat cells** mixed with untreated cells at 0-100% dilution. Calyculin A is a potent phosphatase inhibitor which induces threonine phosphorylation.

Normalization of slides:

- a. Total protein staining by Sypro Ruby: Besides the first and last slides, the middle slide in every 50 slides is stained for total protein with Sypro Ruby. For example, in our printing of 300 slides, we stain the following slides: #1, #50, #100, #150, #200, #250, and #300. The antibody-labeled slides #2-#25 are normalized by #1 total protein slide while antibody-labeled slides #26-#49 and #51-75 are normalized by slide #50 total protein slide, antibody-labeled slides #76-99 and #101-125 are normalized by #100 total protein slide, so on and so forth.
- b. Negative control slides: For each batch staining of up to 33 slides, one slide is stained with antibody diluent instead of a primary antibody and is used as a negative control.