Antibody Array Assay Service Summary

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Assay Protocol

Protein Extraction

1. Wash cells with ice cold 1X PBS.
2. Add Lysis Beads and Extraction Buffer to the sample.
3. Mix rigorously by vortexing for 30 seconds. Incubate the mixture on ice for 10 minutes.
4. Repeat vortexing for 30 seconds at 10-minute intervals for 60 minutes. Incubate the mixture on ice between vortexing.
5. Centrifuge the mixture at 10,000 x g for 20 minutes at 4°C. Transfer the supernatant to a clean tube.
6. Use spin columns to change the buffer in the supernatant to Labeling Buffer.
7. Measure protein concentration.

Protein Labeling

1. Add 100 uL of DMF to 1 mg of Biotin Reagent to give a final concentration of 10 ug/uL.
2. Add Labeling Buffer to the protein sample to bring the volume to 75 uL.
3. Add 3 uL of the Biotin/DMF to the protein sample with Labeling Buffer.
4. Mix and incubate at room temperature for two hours with mixing.
5. Add 35 uL of Stop Reagent. Incubate for 30 minutes at room temperature with mixing.

Coupling

1. Blocking: Submerge Antibody Microarray in Blocking Buffer. Shake for 40 minutes at room temperature.
2. Rinse the slide with Milli-Q grade water.
3. Incubate the slide in Coupling Chamber with 90ug of labeled protein sample in 6 mL Coupling Solution on an orbital shaker for 2 hours at room temperature.
4. Remove the slide from the coupling chamber.
5. Wash the slide three times with fresh Wash Buffer.
6. Rinse extensively with DI water.

Detection

1. Add 30 ul of Cy3-Streptavidin (1 mg/ml) to the 60-ml bottle containing Detection Buffer.
2. Submerge the slide in 30 ml of Cy3-Streptavidin solution.
3. Incubate on an orbital shaker for 45 minutes at room temperature in the dark.
4. Wash the slide three times with fresh Wash Buffer.
5. Rinse extensively with DI water.
6. Dry the slide with compressed nitrogen.
7. Scan on Axon GenePix Array Scanner.

Assay Data Analysis Protocol

Assay data (Worksheet 1)

1. For each spot on the array, median signal intensity is extracted from array image.
2. For each antibody, determine the average signal intensity of replicate spots. The data is labeled as *Average Signal Intensity of Replicate Spots on the Array*.
3. The *CV of the Replicates on the Array* is the coefficient of variation for the replicate spots for each antibody.
4. For normalization, within each array slide the median value of the Average Signal Intensity for all antibodies on the array is determined. This value is presented as *Median Signal*.
   - Normalized data = Average Signal Intensity of Replicate Spots / Median Signal
   - The results are as labeled as *Data Normalized to Median Signal*.
5. Using the normalized data, determine the fold change between control and treatment samples.
   - *Fold change* = Treatment Sample / Control Sample
   - Results are highlighted in different shades of red and green:
     - *Red*: increase in expression
     - *Green*: decrease in expression

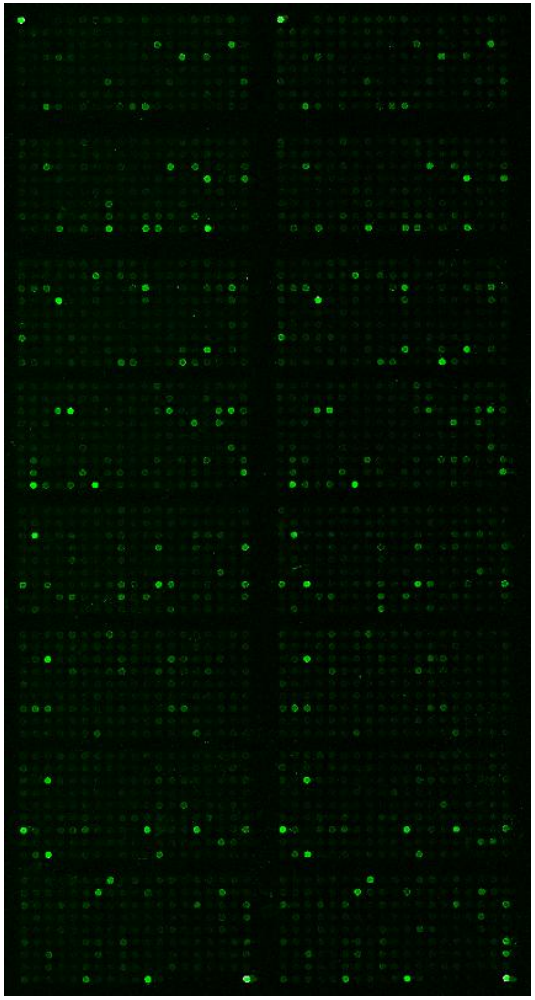
Ratio Analysis (Worksheet 2)

1. Using the *Average Signal Intensity of Replicate Spots on the Array*, for each pair of site-specific antibody and phospho site-specific antibody, the *Signal Ratio* of the paired antibodies is determined.

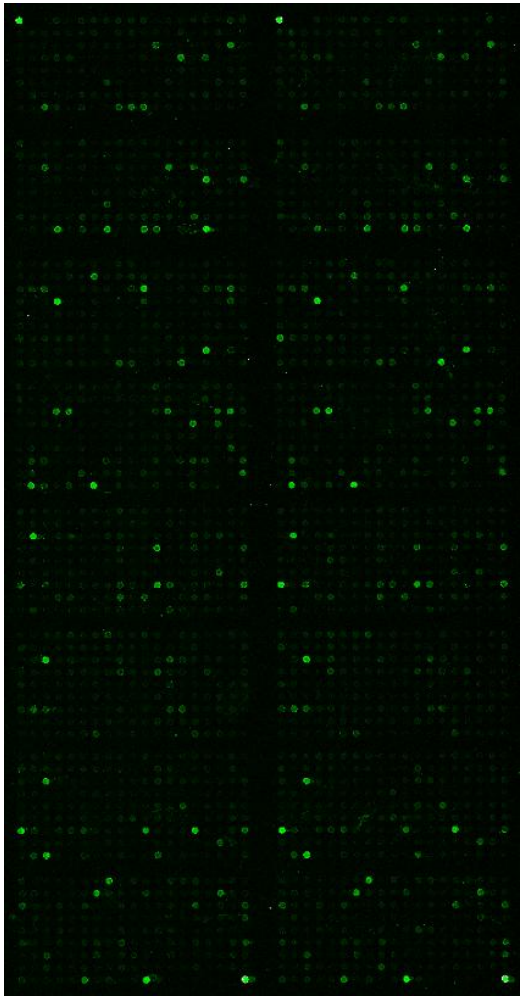
   Ratio = (Signal Intensity of Phospho Site-Specific Antibody) / (Signal Intensity of Site-Specific Antibody)
2. Ratio change between samples = Treatment Sample / Control Sample
   - Results are highlighted in different shades of red and green:
     - *Red*: increase in expression
     - *Green*: decrease in expression

Array Images

Phospho Explorer Array



Control Sample



Treatment Sample

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Data Interpretation

Fold change between samples

• In general, a fold change is considered significant when the value is less than 0.5 or greater than 2. A value of 0.5 indicates that the protein amount has decreased by 50%, and a value of 2 means the protein amount has doubled.

• However, the cut-off value for significant fold change can vary with sample type, treatment method and dosage, and other aspects of the experiment. You should determine the appropriate cut-off value accordingly.

Site-Specific antibody vs. Phospho Site-specific antibody (Phospho arrays only)

• In the phospho antibody arrays, there are two types of antibodies, site-specific antibodies and phospho site-specific antibodies, such as, p53 (Ab-15) and p53 (Phospho-Ser15). The number in the parentheses indicates phosphorylation site. For instance, antibody p53 (Ab-15) is made from a synthetic nonphosphopeptide derived from human p53 around the phosphorylation site of Serine 15. It detects endogenous levels of total p53 protein. Antibody p53(Phospho-Ser15) is made from a synthetic phosphopeptide derived from human p53 around the phosphorylation site of Serine 15. It detects endogenous levels of p53 only when phosphorylated at Serine 15. In most cases, both site-specific antibody and its phospho site-specific antibody pairs are included in the array.

• There may be multiple site-specific antibodies against the same protein. They all detect the same total protein but each recognizes specific residues around the corresponding phosphorylation site. For example, c-Jun (Ab-91) detects total c-Jun protein by recognizing specific residues around phosphorylation site threonine 91; c-Jun (Ab-170) detects total c-Jun protein by recognizing specific residues around phosphorylation site tyrosine 170.

True signal

• A signal is considered a true signal when the signal intensity is at least 2 – 3 times of the background intensity value. In general, the average signal of Empty Spots can be used as background signal.

• When evaluating the data with significant fold changes, it is important to review the signal intensity and make sure the data is derived from true signals.

Data Interpretation

Data analysis method

• There are many different ways to analyze antibody array results. Some methods may produce more meaningful results than others. The method used here is just one of the methods, but it may not be the optimal choice for your sample type and experimental settings. We encourage you to take the signal intensity data and analyze them using other methods.

Proteins not detected

• Because the proteins used in the antibody array assays are not denatured, their tertiary folding structures are intact. As a result, there is an increased chance for false negatives due to inaccessible binding residues.

• Certain proteins form complexes with other proteins immediately after phosphorylation. This phenomenon can block the phosphorylation site from being recognized by the antibodies; as a result, the protein is not detected.

Result verification

• Protein changes derived from antibody array assays should be validated by other methods, such as immunoblotting. Most of the changes can be reproduced by Western blots, but some may not due to the dependency of non-denaturing conditions in antibody arrays.