

Chapter 15

Antibody Specificity Profiling on Functional Protein Microarrays

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Summary

Antibodies represent the end product of an exquisitely complex biological process including recombination, somatic hypermutation, affinity maturation, and self-tolerance, culminating in binding reagents directed against a vast repertoire of antigens. The resultant high affinity and diversity of specificity of these biomolecules has been exploited through the development of immunoassays and biotherapeutics that inaugurated a new era in experimental molecular biology and pharmaceutical drug development. Despite the utility of antibodies for research applications and in disease treatment, they must be employed in the context of an accurate understanding of their binding profile. High-content microarrays comprised of thousands of native, full length human proteins are an important tool in the assessment of antibody specificity.

Key words: Protein microarray, Antibody specificity, Off-target binding, Microarray, Antibody, Proteomics, Protein array.

1. Introduction

Antibodies represent invaluable tools that are critical to a vast array of research applications, and comprise a rapidly expanding segment of the pharmaceutical industry. In research, antibodies are commonly used for affinity purification, protein quantitation, and localization of proteins in tissues or cells. Antibodies are also widely used in diagnostic laboratories for protein quantification. The therapeutic antibody market is expected to nearly triple between 2004 and 2010, with more than 370 programs currently in the pipeline (1). To sustain this rate of growth, antibody developers must take advantage of emerging technologies that

both refine the pipeline and accelerate the development process. Despite the broad utility of antibodies, many methods for determining antibody specificity remain costly and time consuming. However, formal or established methods to rigorously investigate antibody specificity are not currently available, and exceptions to the “one antibody one antigen” rule are often reported only when experimental evidence suggests that an antibody is leading to an unanticipated result due to off-target binding (2–10). While specificity is the hallmark of antibodies, the widely acknowledged potential for antibody cross-reactivity makes interpretation of results in basic research applications more difficult, and increases the potential for unanticipated adverse side effects in clinical applications (4–7, 9–12). Indeed, the importance of precise and thorough characterization of antibody specificity during the development of antibody therapeutics has recently been underscored by the FDA (13).

Clinical trials of therapeutic monoclonal antibodies often reveal side effects that cannot be immediately explained by dose toxicity or by the known protein binding partners. Through the use of protein microarray technology, it is now possible to simultaneously profile thousands of functional proteins to quickly and accurately assess the specificity of any antibody (14–17). Utilizing this technology at an early stage in the therapeutic antibody development process may allow enhanced selection of antibodies that are likely to have improved performance in clinical trials. Protein microarray content comes in many forms, including micro-scale samples of tissue, cells, cellular lysates, or purified antigens (18). While these formats may all have utility in profiling antibody specificity, optimal value is derived from protein microarrays comprised of full length, native proteins with appropriate post-translational modifications. The use of protein microarray technology carries a number of advantages over currently used methods for assessing antibody specificity. The low concentration of many proteins present in cell lysates make western blotting an ineffective approach for comprehensive screening. In addition, the results obtained from western blotting are likely to under-represent antibody cross-reactivity because the denaturing conditions employed in this assay prevent conformational epitope recognition. Therapeutic antibodies in development are commonly surveyed for cross-reactivity through immunocytochemical or immunohistochemical (IHC) methods, and this approach has been extended recently through the use of tissue microarrays. Although tissue microarrays are amenable to higher throughput analysis through the use of automated image processing, the identification of potential cross-reactivity by any IHC-based approach may be limited because of poor antigen availability or by low antigen concentration in a specific cell or tissue sample (19–21). More recently, investigators have utilized mass-spectrometry

to identify cross-reactive antigens following gel electrophoresis or antigen capture by bead-immobilized antibodies (22–24), but these methods remain technically challenging and costly to execute. The sensitivity, reproducibility, and ease-of-use all support the widespread adoption of protein microarray technology for profiling antibody specificity. High-density human protein microarray technology has enormous potential as a tool to markedly accelerate development of optimized antibody research tools and therapeutics.

2. Materials

1. ProtoArray[®] Human Protein Microarray (Invitrogen, No. PAH052402).
2. Quadri-PERM 4-chamber incubation tray (Greiner, Germany).
3. Alexa Fluor[®] 647 goat anti-human IgG (H + L), 2 mg/mL (Invitrogen, Carlsbad, CA).
4. Gene Pix Pro Software (recommended) (Molecular Probes).
5. GenePix 4000B Microarray Scanner (Molecular Probes).
6. Eppendorf centrifuge (5810) (Lab-Line Instruments).
7. Polyacetal slide rack (RA Lamb).
8. Protein Microarrays: Human clones used to produce proteins for ProtoArray[®] Human Protein Microarrays (Invitrogen, Carlsbad, CA) were obtained from Invitrogen's Ultimate[™] ORF (open reading frame) collection or from a Gateway[®] collection of kinase clones developed by Protomatrix. The nucleotide sequence of each clone was verified by full length sequencing. All clones were transferred into a system for expressing recombinant proteins in insect cells via baculovirus infection. Using a proprietary high-throughput insect cell expression system, thousands of recombinant human proteins were produced in parallel. Each protein is tagged with Glutathione-S-Transferase (GST), which enables high-throughput affinity purification under conditions that retain activity. After purification, a sample of every purified protein is checked to ensure that the protein is present at the predicted molecular weight. ProtoArray[®] Protein Microarrays are manufactured using a contact printer equipped with 48 matched quill-type pins. Each protein is deposited along with a set of control proteins in duplicate spots on 1 × 3 inch glass slides that have been coated with a thin layer of nitrocellulose. APiX[™] slides are manufactured by GenTel BioSciences, Inc. The printing of these arrays is carried out in a cold room

under dust-free conditions in order to preserve the integrity of both samples and printed microarrays. Before releasing protein microarrays for use, each lot of slides is subjected to a rigorous quality control (QC) procedure, including a gross visual inspection of all the printed slides to check for scratches, fibers and smearing. Since each of the proteins on the array contains an N-terminal GST tag, a GST-directed antibody detects human proteins in a second QC assay. The procedure measures the variability in spot morphology, the number of missing spots, the presence of control spots, and the amount of protein deposited in each spot. The arrays are designed to accommodate 12,288 spots. For the ProtoArray[®] Human Protein Microarray, samples are printed in 150- μm spots arrayed in 48 subarrays (4,400- μm^2 each) and are equally spaced in vertical and horizontal directions

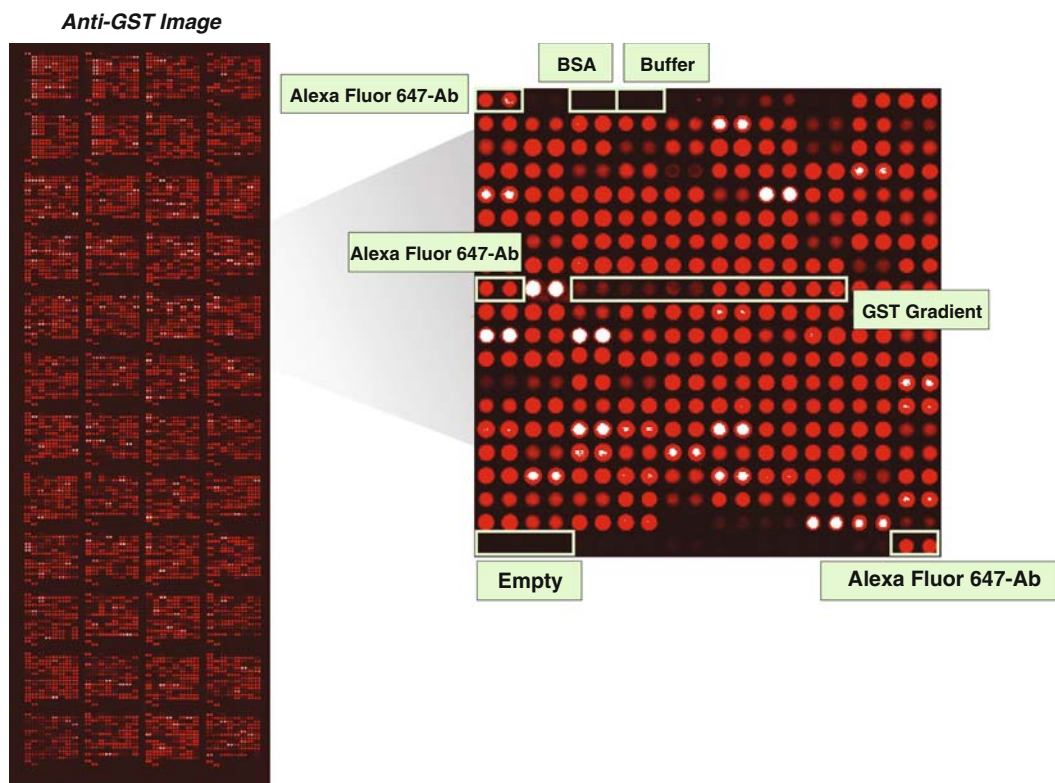


Fig. 1. A ProtoArray[®] Human Protein Microarray was probed with an anti-GST antibody conjugated to Alexa Fluor 647. The microarray was dried and scanned at 635 nm on an Axon 4000B scanner. The ProtoArray[®] is divided up into 48 individual subarrays, each comprised of an identical set of negative and positive control elements, and variable human protein content. An enlarged image of a single subarray is shown on the right, with fluorescent positional mapping markers and a subset of the control elements highlighted. Note the fluorescence pattern associated with the spotting of features as adjacent duplicates (see *Color Plates*).

with 20 columns and 20 rows per subarray. Spots are printed with a 220- μm spot-to-spot spacing. An extra 100- μm gap between adjacent subarrays allows quick identification of subarrays (**Fig. 1**).

9. Protein microarray blocking buffer: 5 mM MgCl_2 , 0.5 mM DTT, 0.05% Triton X-100, 5% glycerol, in PBS, with 1% BSA added fresh prior to assays.
10. Protein microarray PBST probing buffer: 0.1% Tween 20 (w/v) in PBS (Gibco), with 1% BSA (Sigma, protease-free) added fresh prior to blocking.
11. LifterSlip raised edge coverslip (Thermo Fisher Scientific).
12. Secondary Detection Reagent: Alexa Fluor® 647-conjugated anti-species IgG (H + L) at 1.0 $\mu\text{g}/\text{mL}$ diluted in 5-mL probing buffer.

3. Methods

Protein microarrays represent an important new tool in proteomic systems biology. This technology is ideally suited to reveal off-target binding events, and as such provides an approach to facilitate selection of antibody candidates for further development. Additionally, researchers utilizing specific antibodies as reagents in high-throughput assays can employ this technology as a method to rapidly and easily evaluate the quality of these high affinity tools. The method described here assumes the use of ProtoArray® Human Protein Microarrays. The workflow for antibody specificity profiling using protein microarray technology is outlined in **Fig. 2**.

3.1. Protein Microarray Antibody Specificity Profiling Assay

1. Prior to initiating the antibody specificity profiling assay, obtain an appropriate number of protein microarrays for the experimental design (ProtoArray® Human Protein Microarrays from Invitrogen are recommended). Assays are generally performed with two concentrations of antibody over at least a tenfold concentration range. The protein microarrays are stored at -20°C , and must be allowed to equilibrate to 4°C for 10 min prior to initiating the blocking step (*see Note 1*).
2. The blocking buffer is prepared ahead and stored at 4°C , and BSA is added to a final concentration of 1% just prior to use in blocking the protein microarrays. For blocking, protein microarrays are placed protein-side up in a quadriPERM tray, one array per chamber. If the protein microarrays are manufactured on barcoded slides, ensure that the barcode end of

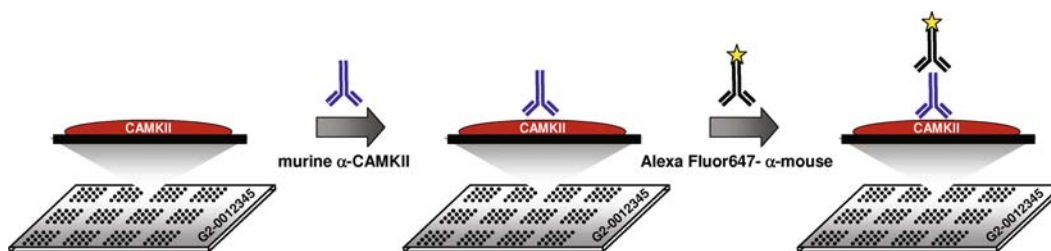


Fig. 2. Protein microarray antibody specificity profiling workflow: Following blocking, the protein microarrays are probed with a dilute solution of the antibody of interest. The antibody binds to its cognate protein on the microarray, and potentially to additional cross-reactive proteins. Following incubation with primary antibody, the microarrays are washed, and incubated with a dye-labelled anti-species antibody. Following incubation with the secondary detection reagent, arrays are washed, dried, and imaged in a fluorescent microarray scanner (*see Color Plates*).

the slide is near the end of the tray with the indented numeral. The indent in the bottom of the tray will be used as the site of buffer exchange. The 4-well trays are gently rocked to ensure that each slide is completely immersed in the blocking buffer. Arrays are blocked for 1 h at 4°C on an orbital shaker in 5-mL blocking buffer per array.

3. During the protein microarray blocking step, the primary antibody solutions are prepared. Antibodies are diluted to suitable concentrations (*see Note 2*) in PBST probing buffer. The probing buffer is prepared ahead and stored at 4°C, and BSA added to a final concentration of 1% just prior to use in probing the protein microarrays. Antibodies can be probed in a total volume of 5 mL. However, if antibody sample is limited, antibody probes can be incubated on the microarray under a LifterSlip in a total volume of 100 μ L.
4. Following blocking, the blocking buffer is aspirated by vacuum or by the use of a pipettor. The tip of the aspirator or pipettor is positioned in the indented numeral in order to remove as much of the liquid as possible. When each well is dry, the indented numeral end of the tray is lifted to facilitate removal of the liquid which pools at the base of the well (*see Notes 3 and 4*).
5. Once the blocking buffer has been completely removed, 5 mL of the dilute antibody solution is added to the quadriPERM tray by pipetting, taking care to avoid pipetting directly onto the array surface. Arrays are then incubated in the primary antibody probing solution for 90 min at 4°C with gentle orbital shaking (*see Note 5*).
6. After probing, the diluted antibody solution is aspirated by vacuum or by the use of a pipettor. The tip of the aspirator or pipettor is again positioned in the indented numeral in order to remove as much of the liquid as possible. When

each well is dry, the indented numeral end of the tray is lifted to facilitate removal of the liquid which pools at the base of the well.

7. The protein microarrays with bound primary antibody are then washed with a series of five 5-min washes in probing buffer. For each wash, 5 mL of probing buffer is added to each well of the tray and allowed to incubate for 5 min with gentle agitation on an orbital shaker before removal by aspiration or pipetting and addition of the next wash.
8. Once the final wash has been completely removed, 5 mL of the dilute secondary detection solution is added to the tray by pipetting, taking care to avoid pipetting directly onto the array surface. Alexa Fluor-conjugated secondary detection reagents diluted to 1 $\mu\text{g}/\text{mL}$ are recommended to maximize signal stability (*see Note 6*). Arrays are then incubated in the secondary detection solution for 90 min at 4°C with gentle orbital shaking.
9. Following the 90 min incubation with the detection reagent, the solution is aspirated by vacuum or by the use of a pipettor. The tip of the aspirator or pipettor is again positioned in the indented numeral in order to remove as much of the liquid as possible. When each well is dry, the indented numeral end of the tray is lifted to facilitate removal of the liquid which pools at the base of the well.
10. The protein microarrays with bound primary and secondary antibodies are then washed with a series of five, five-minute washes in probing buffer. For each wash, 5 mL of probing buffer is added to each well of the tray and allowed to incubate for five minutes with gentle agitation on an orbital shaker before removal by aspiration or pipetting and addition of the next wash.
11. Once the final wash has been completely aspirated, the protein microarrays are removed from the tray and placed in a slide drying rack. To facilitate removal, forceps can be inserted into the indented numeral and used to gently pry the edge of the slide upward. The arrays are then transferred to a slide drying rack with a gloved hand, taking care to only touch the slide by its edges. The arrays are then centrifuged at low speed ($800 \times g$) for one min in a centrifuge equipped with a plate rotor.
12. Following complete drying of the protein microarrays, they are scanned with a fluorescent microarray scanner. The GenePix 4000B scanner set at 635 nm with a laser power of 100% and a focus point of 0 μm is recommended for generating high resolution images (*see Notes 7 and 8*). These images are saved in Tagged Image File Format (.tif-files)

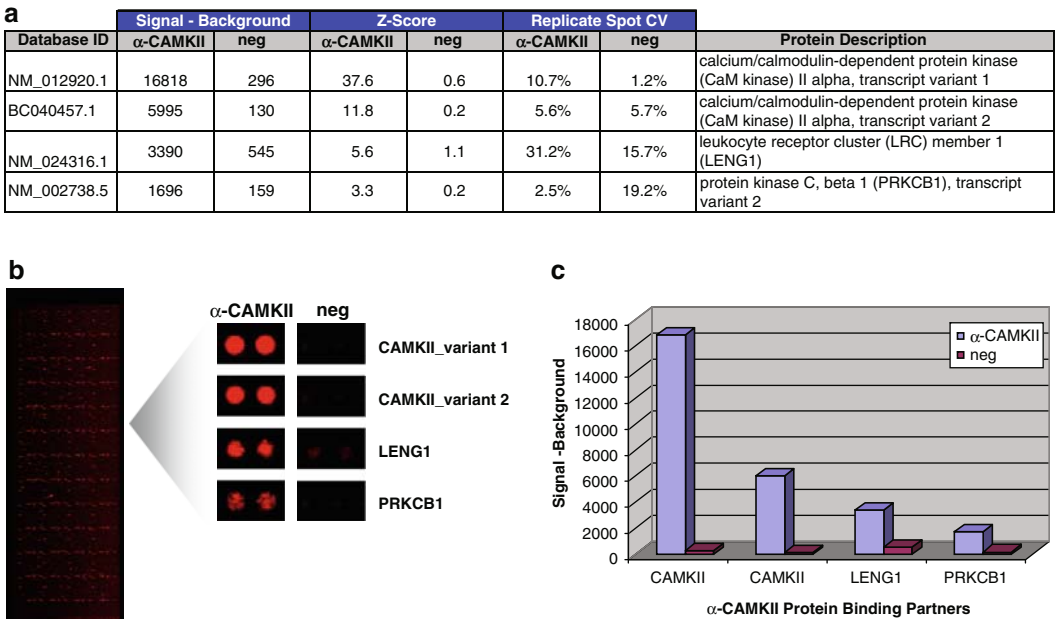


Fig. 3. Protein microarray specificity profiling for anti-CAMKII antibody. **(a)** Murine monoclonal anti-calmodulin kinase II (α -CAMKII, Invitrogen) was probed on a ProtoArray at 1.0 μ g/mL. Following completion of the antibody specificity profiling assay protocol, the arrays were scanned on a fluorescent microarray scanner, and pixel intensity data was used to calculate background subtracted signal values, Z-scores, and coefficients of variation (CV) for adjacent duplicate spots **(b)** The >8,000 human proteins were evaluated for features giving rise to a Z-score >3.0 in the α -CAMKII assay, with a corresponding Z-score <1.5 in the negative control (Alexa Fluor 647-anti-mouse antibody, detection reagent only) assay. Four proteins met these threshold criteria, with the two splice variants of CAMKII identified as the most robust binding partners. Duplicate spots from the α -CAMKII and negative control assays are shown. **(c)** background subtracted signal values are plotted (*see Color Plates*).

and are used in extracting pixel intensity information. As an example, the specificity profile for anti-calmodulin kinase II (CAMKII) generated on a high content protein microarray is shown in **Fig. 3**.

13. Once the protein microarrays are completely dry, store the slides in a light-tight slide box. Prolonged exposure to light will diminish signal intensities. After the arrays have been probed and dried, they can be stored either vertically or horizontally.
14. The array list file (.gal-file) is then uploaded to the image analysis software (GenePix 6.1 from Molecular Devices is recommended). This text file describes the layout of the protein microarray and contains the details of the microarray content, including relevant control elements. The .gal-file is used to map the location of each array features, initially with a fixed feature size based on the diameter of the spotted proteins. To maximize accuracy, a pixel-based segmentation algorithm

is recommended for pixel intensity data extraction (irregular feature finding setting, located under the *Alignment* tab in the *Options* menu of GenePix 6.1) (see **Note 9**). After aligning all features using fluorescent positional mapping markers, pixel intensities for each spot on the array are calculated by the software and saved to a .txt-file formatted for use in GenePix, the GenePix Result file (.gpr-filename extension). These files are subsequently opened in other text editing or spreadsheet programs for analysis.

15. Assuming ProtoArray[®] Human Protein Microarrays are used in the assays, quantified spot files are processed using the ProtoArray[®] Prospector freeware to determine which proteins interact with the antibody probes. The software incorporates background correction, Z-factor and Z-score calculations and replica CV filtering.

4. Notes

1. Use a shaker that keeps the arrays in one plane during rotation. Nutating or rocking shakers are not to be used because of increased risk of cross-well contamination.
2. The choice of antibody concentration should be made in the context of the final working concentration for the antibody. For example, monoclonal or polyclonal research antibodies are commonly probed at concentrations ranging from 0.1 to 1.0 ng/ μ L. Therapeutic antibodies, particularly those administered by subcutaneous injection, are commonly probed at much higher concentrations, in the range of 1–10 mg/mL.
3. Do not aspirate buffers from the surface of the slide in order to reduce the risk of surface scratches.
4. Do not allow any part of the array surface to dry before adding the next solution as this can cause high and/or uneven background.
5. The probe buffer may require modification to increase the stringency of washes in order to minimize background and enhance the detection of high affinity antibody–antigen interactions. Examples of buffer modifications designed to increasing washing stringency include (a) increasing salt (b) increasing amount of detergent during washes (c) increasing temperature during washes or (d) increasing time of washes.
6. The recommended concentration of anti-rabbit/mouse antibody Alexa Fluor conjugate may require optimization to maximize signal to background.

7. During microarray scanning, the PMT should be adjusted such that the feature signals on the array are not saturated. This is done to keep signals within the linear range.
8. For the GenePix 4000B, the best signal-to-noise ratio can be achieved when PMT gain is between 500 and 900. In general, using a PMT gain below 400 is not recommended. If the fluorescence intensity is saturating at full laser power, the laser power should be lowered rather than using an extremely low PMT voltage.
9. In general, the use of pixel-based segmentation (irregular feature finding) results in more reproducible signal used values. The spot diameter and background signal intensities are generally similar between circular and irregular feature finding in antibody-specificity profiling assays.

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