Antibody Profiling on Invitrogen ProtoArray™ High-Density Protein Microarrays

The application of antibodies in research and development, *in vitro/in vivo* diagnostics, or therapeutics requires that antibodies exhibit remarkable specificity. The demand for more specific antibodies to reduce cross-reactivity has increased due to the use of monoclonal antibody therapeutics. Unexpected levels of antibody cross-reactivity can obscure analyses in diagnostic tests or lead to complications during therapeutic antibody regimens in patients. Antibodies typically bind with high affinity (K_d < 50 nM) to their target proteins but vary considerably in specificity. Antibodies exhibiting the highest affinity and specificity are ideal candidates for therapeutics and are more likely to receive regulatory approval (1, 2). An example of such an antibody is Trastuzumab (Herceptin™), a monoclonal antibody produced by Genentech directed against the extracellular domain of Her2, which is used to treat Her2 overexpressing tumors (3). The availability of high-quality antibodies is influenced by the method used for antibody manufacturing and from the assays used to qualify the antibodies.

Various methods for assessing antibody specificity include protein microarrays, Western blotting, ELISA, and immunohistochemistry (4). Compared to other methods, the protein microarray offers several advantages, including rapid screening of thousands of array proteins within a day such that detection is relatively unbiased with respect to protein concentration. In addition, the fluorescent detection method used for the protein array is at least two orders of magnitude more sensitive than the most sensitive chemiluminescent-based detection used for western blotting. Since the identity of every protein printed on the protein array is known, any antibody cross-reactivity with proteins identified on the array can be queried to identify common sequence motifs that might explain cross-reactivity (4). In contrast, antibody specificity profiling using western blots or ELISA is typically slower and the identity of cross-reactive proteins requires follow-up experiments (usually mass spectrometry). Lastly, manipulation of the assay conditions allows one to probe whether epitope recognition on functional protein microarrays requires a structured epitope.

Invitrogen recently introduced the ProtoArray™ Microarray Technology for studying molecular interactions on protein arrays. The ProtoArray™ products include the ProtoArray™ Yeast Proteome Microarray nc 1.0, which contains 4,088 open reading frames (ORFs) from *Saccharomyces cerevisiae*, and the ProtoArray™ Human Protein Microarray nc v1.0, which consists of nearly 1,900 human proteins. All proteins are expressed as N-terminal glutathione S-transferase (GST) fusion proteins, purified, and spotted in duplicate on nitrocellulose-coated 1 inch x 3 inch glass slides. The ProtoArray™ Microarrays provide a convenient method to rapidly screen antibodies of interest for interaction with thousands of proteins within one day.

This Application Note demonstrates the utility of Yeast and Human ProtoArray™ Protein Microarrays in profiling antibody specificity.

Materials and Methods

Yeast Proteome collection: The yeast proteome collection was derived from the yeast clone collection of yeast ORFs generated by the Snyder laboratory as described by Zhu *et al.* (10). Each *S. cerevisiae* open reading frame (ORF) was expressed as an N-terminal GST-6xHis fusion protein in a yeast expression vector. The identity of each clone was verified using 5′-end sequencing and the expression of GST-tagged fusion protein by each clone was confirmed with western immunodetection using an anti-GST antibody. After verifying that each clone expresses a protein of the expected molecular weight, the proteins (from 4,088 clones) were expressed and purified using high-throughput procedures (10).

Human protein collection: The majority of the human protein collection is derived from the human Ultimate™ ORF Clone Collection available from Invitrogen (see http://orf.invitrogen.com for more information). The human proteins were expressed in the Bac-to-Bac® Baculovirus Expression System (Invitrogen Cat. # 10359-016). For more information on the Bac-to-Bac® Baculovirus Expression System, visit www.invitrogen.com. Each Ultimate™ ORF Clone (entry clone) consists of a human ORF cloned into a Gateway® entry vector. Each entry clone was subjected to an LR reaction with the Gateway® destination vector, pDEST™20 to generate an expression clone. The LR reaction mix obtained after performing the LR reaction was transformed into competent DH10Bac™ *E. coli* to generate a recombinant bacmid. The high molecular weight recombinant bacmid DNA was isolated and transfected into Sf9 insect cells to generate a recombinant baculovirus that was used for preliminary expression experiments. After the baculoviral stock was amplified and titered, the high-titer stock was used to infect Sf9 insect cells for expression of the recombinant protein of interest in 96-deep-well plates. Following a 3-day growth, the insect cells were harvested for purification. All steps of the purification process including cell lysis, binding to affinity resins, washing, and elution, were carried out at 4°C. Insect cells were lysed under non-denaturing conditions and lysates were loaded directly into 96-well plates containing glutathione resin. After washing, purified proteins were eluted under conditions designed to obtain native proteins. After purification, samples of the purified proteins were run on SDS-PAGE gels and immunodetected by western blot. The gel images were processed to generate a table of all the protein molecular weights detected for each sample.

ProtoArray™ Manufacturing: The protein purification process described above produces thousands of purified human and yeast proteins ready for printing on microarrays. A contact-type printer equipped with 48 matched quill-type pins is used to deposit each of the purified human or yeast proteins along with a set of control elements in duplicate spots on 1" x 3" nitrocellulose coated-glass slides. The printing of the arrays is performed in a cold room under dust-free conditions to preserve the integrity of protein samples and printed microarrays. Before releasing the protein microarrays for use, each lot of arrays is subjected to a rigorous quality control procedures, including visual inspection of all the printed arrays to check for scratches, fibers, smearing, etc. To control for the quality of the printing process, several microarrays were probed with an anti-GST antibody. Since each protein contains a GST fusion tag, this 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.

Antibody Profiling on ProtoArray™ Microarrays: The Yeast Proteome Microarray nc v1.0 or Human Protein Microarray nc v1.0 was placed in an incubation chamber/hybridization chamber (included in the ProtoArray™ kits) and 20 ml freshly prepared PBST Blocking Buffer (included in the ProtoArray™ PPI Buffer Module from Invitrogen) was applied to the side of the chamber. (It is important not to pipet buffer directly onto the array surface.) The array was incubated with gentle agitation at 4°C for one hour. Primary antibody (20 ml) was prepared in fresh PBS Probing Buffer included in the ProtoArray™ PPI Buffer Module (recommended antibody concentrations are 0.1 µg/ml and 1.0 µg/ml). The PBST Blocking Buffer was decanted from the chamber and primary antibody was applied by pipetting the buffer along the sides of the chamber. The array was incubated with gentle agitation at 4°C for two hours. The primary antibody was decanted and the array washed three times with 20 ml fresh PBS Probing Buffer, approximately 10 minutes per wash, pipetting buffer along the sides of the chamber. The chamber containing the array was inverted briefly on an absorbent surface to completely decant each wash without allowing the array to dry. The final wash was decanted and 20 ml secondary antibody (anti-mouse or anti-rabbit Alexa Fluor® 647 from Invitrogen at 1 µg/ml final concentration) applied in fresh PBS Probing Buffer. The array was incubated with gentle agitation at 4°C for two hours. The secondary antibody was decanted and the array washed three times with 20 ml fresh PBS Probing Buffer, approximately 10 minutes per wash, pipetting buffer along the sides of the chamber. The chamber containing the array was inverted briefly on an absorbent surface to completely decant each wash without allowing the array to dry. The slide was removed from the chamber and tapped on an absorbent surface to remove any residual buffer and placed in a slide box. The array was allowed to dry in the dark with the lid of the slide box open. Slides were completely dried in less than one hour.

For a detailed antibody profiling protocol using ProtoArray™ Microarrays, contact Technical Service (tech_service@invitrogen.com).

Data Acquisition/Analysis: The microarray was scanned with GenePix® 4000B Fluorescent Scanner (Molecular Devices). Data was acquired with GenePix® Pro software (Molecular Devices) and processed using ProtoArray™ Prospector (a software tool developed by Invitrogen that automatically performs data analysis, see www.invitrogen.com/protoarray for details) or Microsoft® Excel and Microsoft® Access.

Statistically significant signals on each protein array were identified. The significant signals are greater than or equal to a value that is determined by calculating the median plus three standard deviations (using signal minus background values for all non-control proteins) for all non-control proteins on the array. Interactors were defined as proteins having positive significance calls not observed on the appropriate negative control.

SDS-PAGE and western analyses of protein samples: Purified proteins were run on SDS-PAGE gels, transferred onto nitrocellulose, and probed with primary antibodies for 2 hours, then probed with secondary antibodies conjugated to HRP for 1 hour. Femto-reagent (Pierce) was used for detection and images were acquired using an Alpha Innotech Imaging station.

Results

Several publications have demonstrated the utility of protein microarrays for profiling antibody specificity (4, 6, 7). Michaud *et al.* (4) showed that antibody quality is highly variable from experiments that involved probing a microarray containing the majority of proteins from the yeast *Saccharomyces cerevisiae* with commercially available antibodies. A yeast protein microarray (ProtoArray™ Yeast Proteome Microarray nc v1.0) was probed with an antibody directed against Hda1, a histone deacetylase important for regulating transcription and chromatin architecture in yeast. In addition to interaction with the cognate Hda1 antigen, seven other proteins exhibited significant signals on the yeast microarray (Figure 1A, left panel). The anti-Hda1 antibody immunogen (a 21 amino acid peptide, TDGLNNIIEERFEEATDFILD) effectively competes with the 7 cross-reactive proteins on the array (Figure 1A, middle panel), while a control peptide sequence (peptide of similar length but unrelated sequence) does not compete (Figure 1A, right panel). This result suggests that the interaction is highly dependent on a specific protein sequence and/or structure. The reactivity of the anti-Hda1 antibody with its cognate protein, Hda1, and three other proteins identified on the array was confirmed by western blot analysis (Figure 1B). No signals were observed after western blot analysis for the other four proteins suggesting insufficient sensitivity of the western blot assay or the presence of a conformation-sensitive epitope that is altered during SDS-PAGE. The presence of protein sequences in the cross-reactive proteins that are similar to the immunogen could explain the apparent cross-reactivity for the seven yeast proteins (Figure 1C).

To assess if similar results would be obtained with antibodies to human proteins, the reactivity of six antibodies (four monoclonal and two polyclonal) was profiled using a human protein microarray (ProtoArray™ Human Protein Microarray nc v1.0) containing approximately 1900 purified human proteins. The cognate antigens for only three of the antibodies were present on the human protein microarray. Each antibody exhibited cross-reactivity with proteins on the array (Table 1). Two of the antibodies (monoclonal Ab Cdk2 and polyclonal Ab Camk1) recognized only their cognate antigens (Figure 2).

Antigens for the anti-Blk, anti-PI3K, and anti-VEGF antibodies are not printed on the array, yet significant cross-reactivity for each antibody was observed: two, four, and one cross-reactive proteins, respectively. The anti-Blk antibody cross-reacted with

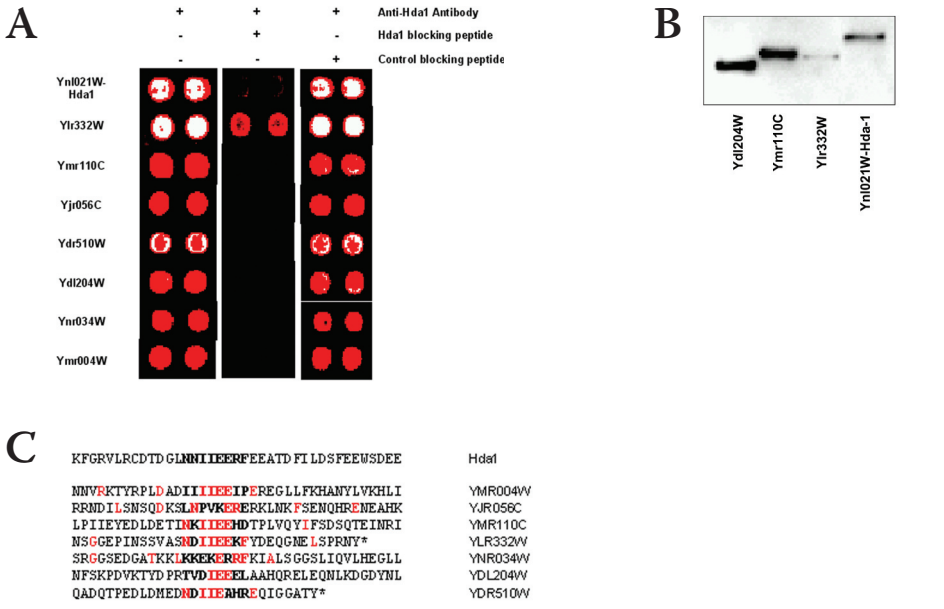

Figure 1—Analysis of anti-Hda1 binding to the Yeast Proteome Microarray. *Panel A* Peptide inhibition of anti-Hda1 binding on the ProtoArray™ Yeast Proteome Microarray showing an array probed with the anti-Hda1 antibody alone (left), an array probed in the presence of the Hda1 immunizing peptide (middle), and an array probed in the presence of a peptide with an unrelated sequence (right). *Panel B* western analysis of proteins detected with the anti-Hda1 antibody on the ProtoArray™ Yeast Proteome Microarray. *Panel C* Amino acid alignments of Hda1 immunogen with cross-reactive proteins (identical residues shown in red).

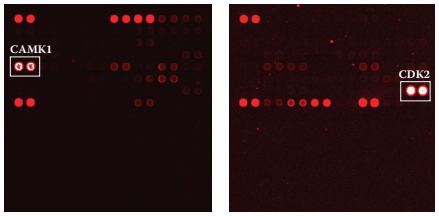

Figure 2—Anti-CAMK1 anti-CDK2 probings of Protoarray™ Human Protein Microarray v1.0. Images for anti-CAMK1 and anti-CDK2 show the antibodies reacting with their cognate proteins. The accession numbers are listed for the cognate antigens. Other signals are from reference spots or from proteins for which signals were not determined to be significant.

proteins FLJ38159 (NM_152723) and C9orf80 (NM_021218). The interactions with FLJ38159 and C9orf80 were further addressed by printing a concentration gradient of these proteins and assessing the antibody reactivity as a function of signal with protein concentration (Figure 3). The difference in signal intensity between the two protein gradients, despite similar protein concentrations, suggests that the affinity of anti-Blk for the two proteins is different.

The anti-Rap1 antibody reacted with five other proteins in addition to the cognate antigen (Table 1/Figure 4A). Several cross-reactive proteins gave even higher signals than were observed for the Rap1 protein (Figure 4A). Note that the cross-reactive proteins yielding higher signals than the cognate protein have approximately 50-fold more protein printed on the array indicating that the cross-reactivity may not reflect a high-affinity interaction. Interestingly, both Rap1 and Rab3C (one of the cross-reactive proteins identified in the array) are members of the ras family of GTP binding proteins. An alignment of Rab3C and Rap1 (Figure 4B) shows considerable sequence similarity and maybe the reason for the observed cross-reactivity.

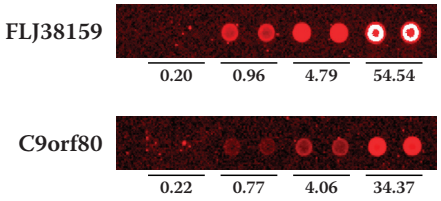

Figure 3—Concentration gradient for anti-Blk cross-reactive proteins. Various amounts of FLJ38159 and C9orf80 were printed on nitrocellulose coated slides and probed with anti-Blk antibody. Numbers beneath each pair of duplicate spots list approximate amount of protein (pmoles) per spot. White signal for FLJ38159 spot is due to signal saturation.

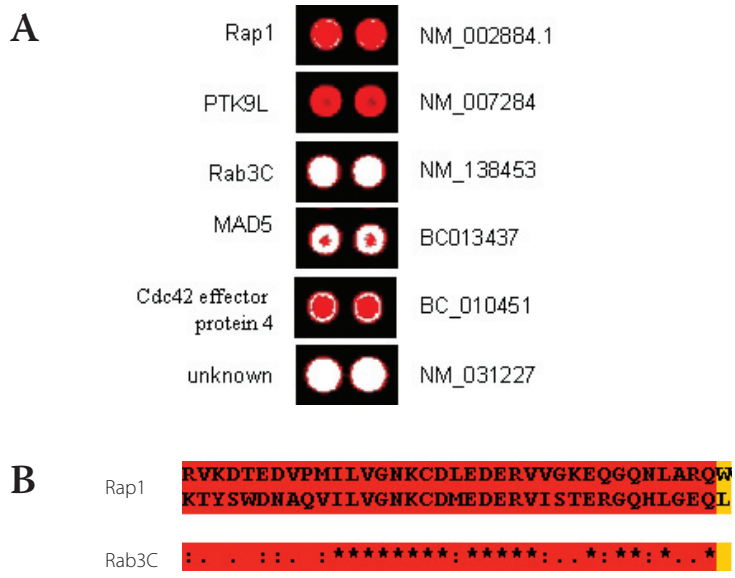

Figure 4—Anti-Rap1 profiling of Protoarray™ Human Microarray v1.0. Panel A Images of anti-Rap1 cross-reactive proteins. The accession number (right) is given for each protein, as well as the common name (left) if known. Also listed are the approximate amounts of protein in pmoles. Rap1 (90pmoles), Cdc42 effector protein 4 (537 pmoles), Rab3C (4660 pmoles), MAD5 (824 pmoles), unknown (3790pmoles). Panel B Sequence alignment of Rap1 and Rab3C (one of the cross-reactive proteins).

Table 1—Antibodies profiled against ProtoArray™ Human Protein Microarray nc v1.0. Four polyclonal and two monoclonal antibodies were purchased from commercial vendors: Blk (Santa Cruz), Rap1 (Santa Cruz), VEGF (Upstate), PI3K (Upstate), CDK2 (Santa Cruz) and CAMK1 (Santa Cruz). The amount of cognate protein (pmoles) printed on the array is listed. The number of cross-reactive proteins for each antibody is determined by defining the signals that are at least three standard deviations over the median signal minus background value for all human proteins (excluding controls) on the array.

Antibody	Accession number for cognate protein	Amount of cognate protein on array (pmoles)	Source of epitope	Immunogen	Antibody type	Antibody concentration (µg/ml) used for probing	Number of cross-reactive proteins identified on the array
Blk	N/A	N/A	mouse	peptide	polyclonal	0.05–0.5	2
Rap1	NM_002884.1	90	human	peptide	polyclonal	0.1–1.0	5
VEGF	N/A	N/A	human	protein	monoclonal	0.1–1.0	4
PI3K	N/A	N/A	human	peptide	polyclonal	0.1–1.0	1
CDK2	BC003065.1	4690	human	protein	monoclonal	0.1–1.0	0
CAMK1	NM_003656.3	4850	human	peptide	polyclonal	0.05–0.5	0

Summary

Cross-reactivity was observed for both polyclonal and monoclonal antibodies using protein microarrays for antibody profiling. We have observed that typically polyclonal antibodies cross-react with more proteins on protein microarrays, whereas monoclonal antibodies exhibit a greater degree of specificity. In some cases, the observed cross-reactivity on the array could be explained by sequence similarity with the cognate protein. However, the observed cross-reactivity could not be predicted by sequence similarity, as many proteins on the array predicted to cross-react did not exhibit any cross-reactivity. These results show the utility of protein microarrays for screening antibody specificity. Combined with the speed, ease of use, coverage, protein information, and high sensitivity, antibody profiling on protein microarrays is likely to become the assay of choice for qualifying antibody specificity for effective development of antibody therapeutics. The use of protein microarrays for antibody profiling aids in the identification of epitopes/antigens for antibodies whose targets have not yet been characterized

References

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