Protein-Protein Interaction Profiling on Invitrogen ProtoArray™ High-Density Protein Microarrays

A powerful means of determining the function of a protein is to map its interactions with other proteins. A variety of approaches are available to study protein-protein interactions, including mass spectroscopy, and yeast two-hybrid methods (1). Yet these technologies have several drawbacks: they are time-consuming, require expensive and specialized equipment as well as considerable expertise to run the equipment, and utilize large amounts of sample. Several large-scale efforts to map protein-protein interactions using mass spectroscopy or yeast two-hybrid have been performed recently (2, 3). Interestingly, a comparison of the results of these studies shows little overlap between the interactions observed in each, suggesting that the accuracy or the coverage of the methods may be lacking (4).

Protein microarrays have introduced a new approach to identify and characterize protein interactions, providing the ability to rapidly identify new interactions between thousands of proteins in a single experiment (5). Since the location and identity of each protein on the array is known, interaction maps can be developed rapidly from iterative probings of protein arrays. Because a protein microarray experiment is performed within a day, and interactions are assessed in the context of thousands of other proteins, interaction profiling on microarrays can greatly accelerate the rate at which novel protein interactions are discovered. Additionally, the *in vitro* nature of protein microarray experiments permits control over probing conditions that affect protein interactions such as protein concentration, post-translational modifications, and presence of cofactors, which may not be possible with other methods such as yeast two-hybrid screening.

MacBeath and Schreiber were among the first to demonstrate the potential of protein microarrays in protein-protein interaction, biochemical, and drug binding studies. In this study, pairs of proteins that were known to interact with each other—protein G and the immunoglobulin (IgG), p50 and IκKα, and the FKBP12 binding domain of FKBP with the human immunophilin FKBP12—were shown to interact on protein microarrays (6). Although this study represented a critical milestone in the development of functional protein arrays, only a few proteins were analyzed and novel activities were not identified. Since this report, a series of publications have demonstrated that proteins can retain their expected interactions while immobilized on microarray surfaces. Espejo *et al.* demonstrated that protein interaction domains, such as Src homology (SH2), 14.3.3, forkhead-associated (FHA), PDZ, pleckstrin homology (PH), and FF domains arrayed onto nitrocellulose-coated microarrays retain function and specificity, interacting with their corresponding ligands (7). Newman and Keating have used microarrays to characterize binary coiled-coil interactions from human basic-region leucine zipper transcription factors (8). More recently, Ramachandran *et al.* used protein microarrays to map pairwise interactions among several human DNA replication initiation proteins (9). Finally, in what may be the most striking example of the power of protein microarrays, Michael Snyder and colleagues at Yale University reported the fabrication of an array containing the majority of proteins from the yeast proteome and the use of this array to identify a new binding motif for calmodulin (10).

Invitrogen ProtoArray™ Products

Invitrogen has recently introduced the ProtoArray™ Microarray Technology for studying molecular interactions on protein arrays. The ProtoArray™ products include the ProtoArray™ Yeast Proteome Microarray nc v1.0, which contains 4088 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. Using ProtoArray™ Microarrays allows screening of target proteins of interest for interaction with thousands of proteins in as little as four hours. Detection on the arrays is sensitive—as little as 1 pg protein on the array can be detected with submicrogram quantities of probe protein—and reproducible.

To detect protein-protein interactions on ProtoArray™ Microarrays, the protein probe must contain a label or tag to visualize the interaction of the probe with array proteins. The extremely high affinity of the biotin-streptavidin interaction makes biotin-protein conjugation a preferred method for protein labeling. Invitrogen offers the ProtoArray™ PPI Complete Kit for biotinylated proteins, which contains a module for efficiently biotinylating small amounts of a protein as well as qualified reagents for blocking, washing, and detecting biotinylated protein probes with streptavidin conjugated to a fluorescent dye, Alexa Fluor® 647.

Another preferred method of detecting protein interactions on ProtoArray™ Microarrays is to use protein probes with an epitope tag and a labeled antibody against the tag. An example of such a tag is the V5 epitope, a 14 amino acid (GKPIPNPLLGLDST) epitope derived from the P and V proteins of the paramyxovirus SV5. Invitrogen offers several Gateway® expression vectors that allow the fusion of the V5-tag to a protein of interest. The ProtoArray™ PPI Complete Kit for epitope-tagged proteins from Invitrogen provides reagents for blocking, washing, and detecting a V5-tagged protein using an Anti-V5-Alexa Fluor® 647 Antibody developed specifically for this application.

This Application Note demonstrates the utility of Yeast and Human ProtoArray™ Protein Microarrays for detecting protein-protein interactions using the biotinylated or epitope-tagged protein probes.

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. no. 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 wass 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 are 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 in 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 proteins ready to be printed on arrays. A contact-type printer equipped with 48 matched quill-type pins is used to deposit each of these proteins along with a set of control elements in duplicate spots on 1″ x 3″ glass slides. The printing of these 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 are 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.

Cloning, Expression, and Purification of Proteins (6xHis-V5-BioEase™-EK-protein fusions): Ultimate™ ORF clones were obtained as entry clones and L x R cloned into pET105 for expression in *E. coli.* For each ORF, plasmid DNA was transformed into BL21 Star™ (DE3) *E. coli* cells, which were plated on LB/Amp and grown overnight at 37°C. Several colo-

nies from each of the 12 constructs were picked from LB/Amp plates and transferred into 50 ml of LB Amp. Cultures were grown from 5 to 7 hours at 37°C until an OD_{600} of 0.5 to 0.6 was reached. Next, 50 µl of 0.1 M IPTG was added to give a final concentration of 100 µM, and these cultures were incubated overnight at 20°C. Cell lysates were prepared using the protocol described in the ProBond™ Purification Resin manual. Pellets were resuspended with 8 ml Native Binding Buffer; 8 mg lysozyme was added and lysed for 30 minutes on ice. Cells were then sonicated on ice with six 10-second bursts and then centrifuged at 3,500 rpm for 20 minutes. Lysate (8 ml) was loaded onto a column with 2 ml washed ProBond™ resin and incubated for 1–2 hours at 4°C. The column was washed with Native Wash Buffer followed by an elution with 10 ml Elution Buffer. The pooled fractions were dialyzed twice against 2 L PBS. All samples were concentrated on Millipore spin membrane cartridges (10,000 MW cut-off) to a final volume of 250–350 µl, and were brought to 5% glycerol by the addition of an appropriate amount of 100% glycerol. Samples were then quick-frozen in liquid nitrogen and stored at –80°C.

***In vitro* biotinylation of proteins:** Human calmodulin (Upstate) was biotinylated using the protocol outlined in the ProtoArray™ Mini-Biotinylation Kit (Invitrogen). Briefly, protein was biotinylated at room temperature for 1 hour and the sample was applied to a gel filtration column to remove unincorporated biotin. Protein concentration and the extent of labeling was also assessed.

Alexa Fluor® 647-streptavidin based detection: The protein-protein interaction assay was performed using the protocol outlined in the ProtoArray™ PPI Complete Kit for biotinylated proteins (Invitrogen). Arrays were blocked with 1% BSA/PBST at 4°C for 1 hour. Proteins were diluted in probe buffer (1X PBS, 5 mM $MgCl_2$, 0.5 mM DTT, 5% glycerol, 0.05% Triton X-100, 1% BSA) to 5 or 50 ng/µl and added to arrays under a cover slip, Hybrislip (included in the kit). Proteins were incubated at 4°C for 90 minutes in a 50 ml conical tube and then transferred to an incubation/hybridization chamber (included with the kit). Arrays were washed three times with probe buffer. Subsequently, a solution of Alexa Fluor® 647-streptavidin (Invitrogen, 0.75 µg/ml) in probe buffer was added and incubated at 4°C for 30 minutes. Arrays were washed three times and dried.

Anti-V5-Alexa Fluor® 647 based detection: The protein-protein interaction assay was performed using the protocol outlined in the ProtoArray™ PPI Complete Kit for epitope-tagged proteins (Invitrogen). Arrays were blocked with 1% BSA/PBST at 4°C for 1 hour. Proteins were diluted in probe buffer to 5 or 50 ng/µl and added to arrays under a Hybrislip cover slip. Proteins were incubated at 4°C for 90 minutes in a 50 ml conical tube and then transferred to an incubation/hybridization chamber (included in the kit). Arrays were washed three times with probe buffer. Subsequently, a solution of anti-V5-Alexa Fluor® 647 conjugated antibody (Invitrogen, 0.25 µg/ml) was added and incubated at 4°C for 30 minutes. Arrays were washed three times and dried.

Data acquisition/analysis: The microarray was scanned with a 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.

Results

Probing ProtoArray™ Yeast Proteome Microarrays with biotinylated yeast proteins: Four yeast proteins were biotinylated *in vitro* using the Invitrogen ProtoArray™ Mini Biotinylation Kit. As shown in Figure 1, all four proteins showed expected interactions when used to probe the ProtoArray™ Yeast Proteome Microarray and detected with Alexa Fluor® 647-Streptavidin Conjugate. Each of the identified interactions is well annotated in the literature using a variety of different approaches (see http://www.yeastgenome.org for further details). Note that the interactions shown in Figure 1 are reciprocal. Biotinylated Ybr109C (calmodulin) interacts with Yfr014C (calmodulin kinase) on the array, and biotinylated Yfr014C interacts with Ybr109C on the array; the same relationship is observed with the GTP binding protein

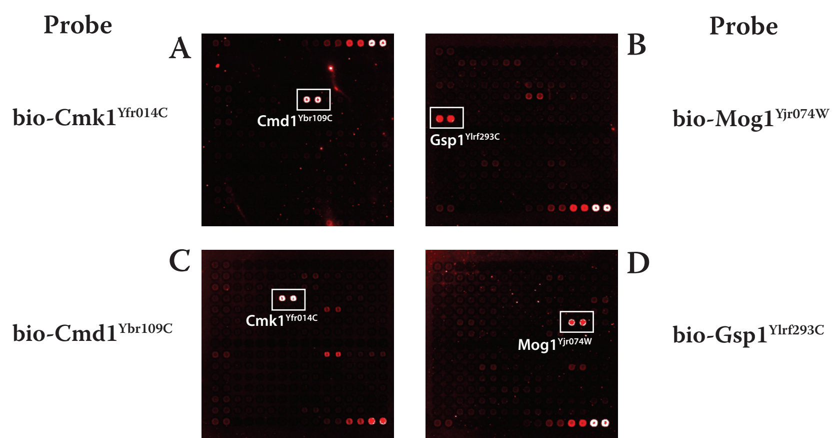


Figure 1—Probing the ProtoArray™ Yeast Proteome Microarray with *in vitro* biotinylated yeast proteins. Subarrays show expected interactions with biotinylated yeast proteins. Proteins were concentrated to 250 µg/ml and biotinylated using the ProtoArray™ Mini-Biotinylation Kit.

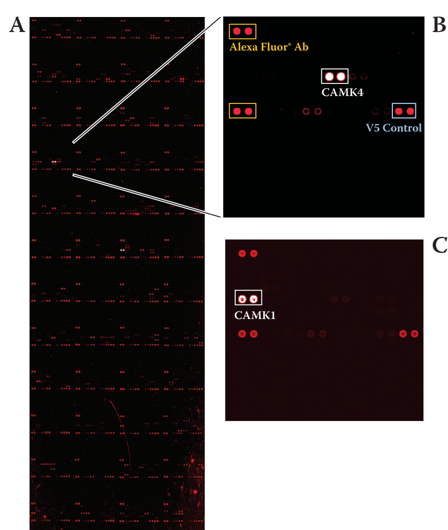
Gsp1 (Ylr293C) and the nuclear transport protein Mog1 (Yjr074W). The reciprocal interactions are important for demonstrating the validity of the observed interactions and the functionality of the proteins on the array.

Probing ProtoArray™ Human Protein Microarrays with Biotinylated and Epitope-tagged Human Proteins: To assess the utility of human protein arrays and protein-protein interaction detection technologies optimized at Invitrogen for demonstrating protein-protein interactions, proteins containing both a single biotin and a V5 tag were prepared (see Materials and Methods). Several N-terminal fusions of V5-BioEase™ human proteins were probed against human protein arrays (ProtoArray™ Human Protein Microarray nc v1.0) consisting of approximately 1,900 purified human proteins spotted in duplicate on a nitrocellulose-coated glass slide. After probing the array with calmodulin 2 (CALM2), we observed that CALM2 interacted with several proteins on the array. Most notable are the interactions with calcium/calmodulin-dependent protein kinase IV (CAMK4) and calcium/calmodulin-dependent protein kinase I (CAMK1) (Figure 2). These interactions were observed when streptavidin (data not shown) or anti-V5 based detection was used (Figure 2). We also used the ProtoArray™ Mini-Biotinylation Kit to *in vitro* biotinylate recombinant human calmodulin and used this protein to probe the ProtoArray™ Human Protein Microarray nc v1.0. As shown in Figure 3, similar protein interactions with CAMK1 and CAMK4 were observed for *in vitro* biotinylated calmodulin as with the BioEase™-tagged CALM2, demonstrating that valid protein-protein interaction data can be obtained by using proteins that are biotinylated using *in vitro* or *in vivo* methods.

To demonstrate the utility and ease of use of ProtoArray™ Technology for identifying novel protein-protein interactions, a V5-BioEase™ fusion to the protein cyclin-dependent kinase inhibitor 1B (CDK1NB, p27, Kip1) was used to probe a ProtoArray™ Human Protein Microarray. We identified a specific interaction with cyclin-dependent kinase 7 (Cdk7, MO15 homolog, *Xenopus laevis*, cdk-activating kinase) (Figure 4). The same interaction was also observed using streptavidin-based detection (data not shown). Although this interaction has not been reported previously in the literature, an interaction of CDK1NB with Cdk3 has been reported, and it has been proposed that retinoic acid induces cell cycle arrest in tumor cell lines by promoting formation of this complex (11). To validate the interaction, we performed the following reciprocal protein-protein interaction assay: CDK1NB was spotted on a nitrocellulose coated slide, then probed with GST-tagged Cdk7, and the Cdk7-CDK1NB complex was detected using an anti-GST antibody. Similar probings with 18 other GST-tagged proteins gave signals with the spotted CDK1NB that were on average approximately 10-fold lower than Cdk7 (Figure 5), indicating that the Cdk7-CDK1NB interaction is quite specific.

Summary

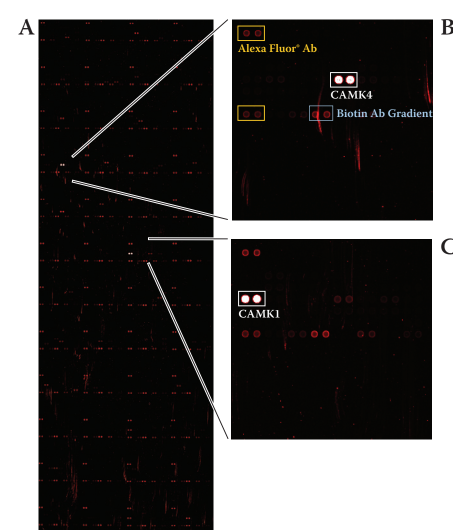
ProtoArray™ Protein Microarrays with Alexa Fluor® detection technologies are optimized to quickly identify novel protein-protein interactions. High-quality reagents, protocols and technical support are available. Consult the Invitrogen website for the latest information regarding protein microarrays for protein interaction profiling using ProtoArray™ Technology.



◀ **Figure 2—ProtoArray™ Human Protein Microarray nc v1.0 probed with human CALM2.** Interactions detected with anti-V5-Alexa Fluor® 647 Dye.

Panel A Whole slide image.

Panel B Interaction of CALM2 with CAMK4. Signals from Alexa Fluor® Antibody and V5 control are shown. Alexa Fluor® labeled antibody is in every subarray and used as a reference marker for aligning the data acquisition grid. The V5 control is a V5 tagged protein printed on the slide. Signal with this protein indicates that assay detection is functioning properly.



▶ **Figure 3—ProtoArray™ Human Protein Microarray nc v1.0 probed with *in vitro* biotinylated human calmodulin.** Interactions detected with streptavidin Alexa Fluor® 647 Dye.

Panel A Whole slide image.

Interactions of human calmodulin with CAMK4 (*Panel B*) and CAMK1 (*Panel C*). Signals from Alexa Fluor® Antibody and biotinylated antibody gradient are shown. The biotinylated antibody gradient is used as assay detection control. Signal with this protein indicates that assay detection is functioning properly.

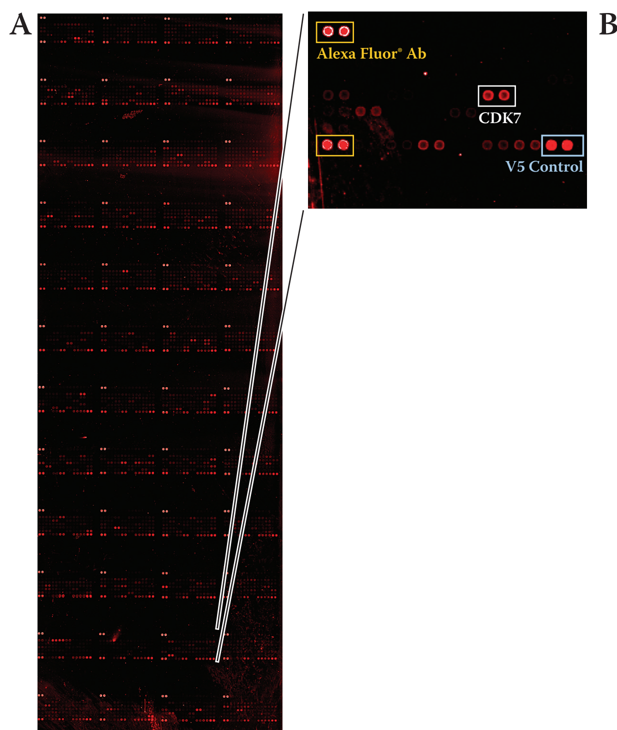


Figure 4—ProtoArray™ Human Protein Microarray nc v1.0 probed with CDKN1B. Interactions detected with anti-V5-Alexa Fluor® 647 Dye. *Panel A* Whole slide image. *Panel B* Interaction of CDKN1B with CDK7. Signals from Alexa Fluor® Antibody and V5 control are shown.

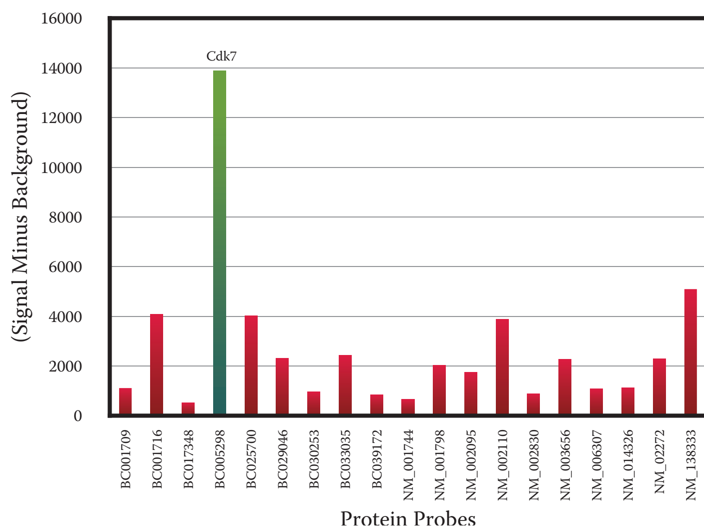


Figure 5—Reciprocal Protein Interaction Assay. Nineteen GST-fusions were expressed in Sf9 cells, purified using glutathione chromatography, and probed against an array containing immobilized CDKN1B. The Y-axis is the signal background value for the CDKN1 spot for each protein probed (X-axis) against the array. The accession numbers (MGC or RefSeq) for the protein probes are listed (X-axis). The MGC accession number for Cdk7 is BC005298. CDKN1B was spotted at an equivalent solution protein concentration of approximately 12 ng/μl. The median probing concentration for the 19 proteins was 11 ng/ul; the mean concentration was 12 ng/μl.

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