

Biomarker Discovery: Immune Response Profiling on ProtoArray[®] Human Protein Microarrays

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Introduction

Molecular profiling at the transcript level and, more recently, at the protein level offers a more rapid and high-throughput systems biology approach to scientific and medical discovery compared to traditional single-protein or single-pathway studies. Emerging technologies providing global profiling solutions, particularly in the area of biomarker discovery, represent exciting opportunities for the biomedical research community. Biomarker panels usually allow early diagnosis, aid in prognosis, and facilitate selection of treatment and disease monitoring.

The use of antibodies and/or their antigen targets as biomarkers can greatly facilitate the development of therapeutics and diagnostics for a variety of infectious diseases, cancers, and autoimmune disorders. A classic example of this approach is the Her2 protein, a member of the epidermal growth factor family of receptor tyrosine kinases, which is known to be overexpressed in approximately 30% of breast cancers. The expression profile identified Her2 as an important therapeutic target, resulting in the development of the blockbuster drug Herceptin[®] (trastuzumab), a humanized monoclonal antibody that induces downregulation of Her2 through specific targeting of the extracellular domain (1). Another biomarker, NY-ESO-1 protein, is known to stimulate a humoral response in a variety of cancers including melanoma (2). Serum antibodies to the NY-ESO-1 protein have been detected in NY-ESO-1–positive cancers by western blot analysis and ELISA (enzyme-linked immunosorbent assay) (3). Autoimmune diseases such as systemic lupus erythematosus (SLE) and rheumatoid arthritis (RA) are also characterized by the presence of antibodies directed against autoantigens (self proteins). The symptoms and severity of autoimmune diseases often correlate with the presence of autoantibodies and the localization of the autoantibody-autoantigen complexes within the body. The presence of autoantibodies against several autoantigens in the sera of

patients prior to clinical diagnosis of disease has also been demonstrated (4). A more detailed analysis of the development and evolution of the autoantibody profile would allow better screening, diagnosis, and prognosis for patients with autoimmune diseases.

Methods that provide high sensitivity and broad protein coverage, such as two-dimensional gel electrophoresis coupled with mass spectrometry, ELISA, gene expression profiling, and antibody arrays, have been used to identify protein biomarkers in autoimmune disorders, infectious diseases, and cancers (5-7). However, each of these methods has drawbacks that limit its effectiveness for high-throughput biomarker protein analysis from serum. Mass spectrometry-based approaches require dedicated instrumentation, considerable technical expertise, and large amounts of sample, while ELISA assay development requires some prior information about the potential biomarkers. Gene expression profiling on DNA microarrays has limited usefulness due to the discordance between RNA and protein levels (8). Profiling serum proteins on antibody arrays also has drawbacks, including the cumbersome protocol and the difficulty associated with identifying differences in protein levels between two samples, caused by the limited number and variable quality of antibodies present on the array.

More recently, protein microarrays comprised of lysates, protein fractions, or highly purified proteins have been successfully used to profile serum antibody levels in various diseases (9-12). The discovery of protein-based biomarkers is a central aim of proteomic systems biology, and is a critical component for enabling personalized medicine.

Invitrogen ProtoArray® product line

The ProtoArray® Protein Microarray from Invitrogen allows analysis of large numbers of proteins in an easy-to-use format. The ProtoArray® Human Protein Microarray v4.0 consists of approximately 8,000 highly purified full-length human proteins expressed in insect cells as N-terminal glutathione S-transferase (GST) fusion proteins, purified under native condition, and spotted in duplicate on glass slides (1 inch x 3 inch). The ProtoArray® Microarray allows the screening of thousands of target proteins for interactions with serum antibodies, within a single day. Since all proteins on the array are purified under native conditions, the immobilized proteins are expected to maintain their native conformations and therefore should be recognized by antibodies requiring structured epitopes (13). The Invitrogen ProtoArray® Microarray has also been used for a variety of applications including immune response profil-

ing (see www.invitrogen.com/protoarray for the latest applications) (14-16). This application note demonstrates the utility of the ProtoArray® Human Protein Microarrays for immune response profiling that characterizes a patient population of interest.

Materials and methods

Purified proteins and human sera: Purified NY-ESO-1 protein, a mouse monoclonal anti-NY-ESO-1 antibody, human sera from healthy controls, and human sera from patients with melanoma and lung cancer were generously provided by Dr. Ruth Halaban (Yale University) and Dr. Gerd Ritter (Ludwig Institute for Cancer Research at Memorial Sloan-Kettering Cancer Center).

Human protein collection: Nearly all of the human protein collection printed on the ProtoArray® Microarray is derived from the human Ultimate™ ORF (open reading frame) Clone Collec-

tion available from Invitrogen (see orf.invitrogen.com for more information). Each Ultimate™ ORF clone is full insert–sequenced and is guaranteed to match the corresponding GenBank amino acid sequence. (For accession number and amino acid sequence for each protein, download the Protein Information File from www.invitrogen.com/protoarray.)

All clones used to generate the human protein collection were entry clones constructed from a human ORF cloned into a Gateway® entry vector. Each entry clone was subjected to a LR recombination reaction with the Gateway® destination vector, pDEST™20, to generate an expression clone. The expression clone was then used to express the protein in Sf9 insect cells as an N-terminal GST fusion protein using the Bac-to-Bac® Baculovirus Expression System available from Invitrogen. The GST-tagged fusion proteins are purified using high-throughput procedures that have been optimized to obtain maximal protein integrity, function, and activity. Each protein is checked by western blotting to verify that it has the expected molecular weight.

ProtoArray® manufacturing: The purified human proteins were printed on nitrocellulose-coated glass slides in a dust-free, temperature- and humidity-controlled environment to maintain consistent quality of the microarrays. The arrays were printed using an automated process on an arrayer that is extensively calibrated and tested for printing ProtoArray® Human Protein Microarrays. After production, each microarray was visually inspected for obvious defects that could interfere with the experimental results. To control for the quality of the printing process, several microarrays from each lot were probed with an anti-GST antibody. Since the proteins contain a GST fusion tag, probing the microarrays with an anti-GST antibody allows identification of irregular spot

morphology or missing spots. The arrays were also functionally qualified by probing for control proteins to detect the appropriate interactions.

Serum Profiling Assays on ProtoArray® Protein Microarrays:

Arrays were blocked with Blocking Buffer (1% BSA, 1X PBS, 0.1% Tween® 20) at 4°C for 1 hour. Serum samples were diluted in Probe Buffer (1X PBS, 5 mM MgCl₂, 0.5 mM DTT, 5% glycerol, 0.05% Triton® X-100, 1% BSA) and added to arrays under a LifterSlip™ cover slip. The arrays were incubated at 4°C for 90 minutes in a covered chamber such as a petri dish and then transferred to an incubation/hybridization chamber. Arrays were washed three times, 8 minutes per wash with gentle shaking using 20 ml Probe Buffer. Subsequently, a 1.0 µg/ml solution of anti-human IgG conjugated to Alexa Fluor® 647 (anti-Human IgG-Alexa Fluor® 647 from Invitrogen) in Probe Buffer was added and incubated at 4°C for 90 minutes. Arrays were washed three times (as above) and dried.

Data Acquisition/Analysis: Arrays were scanned with a GenePix® 4000B fluorescent scanner (Molecular Devices). Data were acquired with GenePix® Pro software (Molecular Devices) and processed using ProtoArray® Prospector 4.0 (a free software tool developed by Invitrogen; for details see www.invitrogen.com/protoarray).

Results

Establishing serum profiling proof-of-principle using custom protein arrays

Figure 1 illustrates the protocol for using the ProtoArray® Human Protein Microarray to characterize the autoimmune profile in a biological sample. Briefly, the protein arrays are blocked with a non-specific protein blocker, incubated with a diluted serum sample,

washed, and then incubated with a secondary reagent to detect bound serum antibodies. In the experiments described below, the secondary reagent was an Alexa Fluor®647–conjugated anti–human IgG antibody that interacts with the Fc portion of serum IgG. To demonstrate the utility of ProtoArray® technology for the identification of protein biomarkers, we probed custom protein arrays containing a gradient (approximately 0.4–100 pg) of NY-ESO-1 protein with a serum sample known to be sero-reactive against NY-ESO-1 protein. A 1:150 dilution of the serum sample produced a readily detectable signal with as little as 0.4 pg of NY-ESO-1 protein (Figure 2). Significant signals were not observed with a negative control array containing purified GST (100 pg protein). Parallel probing of arrays with increasing dilutions of the serum sample (1:150, 1:1,000, 1:5,000, 1:10,000, and 1:50,000) revealed a concentration dependence between the signal and serum concentration. Readily detectable signal was observed with serum dilutions up to 1:50,000, on arrays containing as little as 25 pg of recombinant NY-ESO-1 protein. These results demonstrate that serum profiling for detection of human antibody-protein complexes on protein arrays is highly specific and sensitive.

To evaluate the reproducibility of the immune response profiling application, the ProtoArray® Human Protein Microarray v1.0 containing approximately 2,000 different human proteins was assayed in duplicate with serum samples from a healthy donor and a melanoma patient. A panel of values was calculated for each human protein spot across the replicates, including the coefficient of variation, R^2 , and Pearson's Correlation Coefficient. As shown in Table 1, signal intensity values derived from ProtoArray® immune response profiling experiments are highly reproducible.

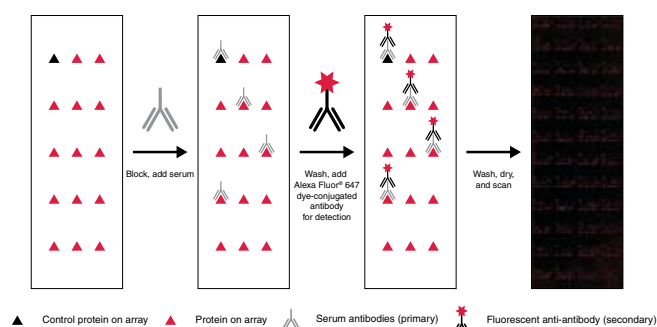


Figure 1—Protocol for serum profiling application. Briefly, the ProtoArray® Human Microarray is blocked with a nonspecific protein blocker, treated with diluted serum, and incubated for 90 min. The array is washed to remove any unbound proteins/antibodies. The bound serum antibodies are then detected with anti-isotype-specific secondary antibodies conjugated to Alexa Fluor® 647.

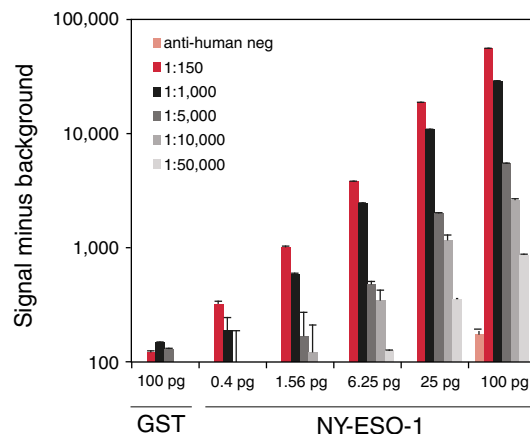


Figure 2—Sensitivity of detection. Signals obtained with of custom protein arrays containing a gradient of NY-ESO-1 protein probed with a dilution series of serum (see Materials and Methods) known to be NY-ESO-1 seropositive. Another array printed with NY-ESO-1 protein, was probed with only the secondary detection reagent (anti-human neg). An array printed with purified GST was used as another type of negative control.

Results Consistent with ELISA

NY-ESO-1 custom protein arrays were also probed with a panel of five serum samples derived from melanoma patients. The seroreactivity of the samples (Mel_1 to Mel_5) against the NY-ESO-1 protein had been previously established by ELISA at an independent laboratory. Sera included one sample identified by ELISA as NY-ESO-1 seropositive (Mel_1), and another identified by ELISA as NY-ESO-1 seronegative (Mel_2). The remaining three serum samples were blinded. As shown in Figure 3, the known seronegative (Mel_2) and seropositive (Mel_1) samples gave the expected signals on the arrays. Results of the three blinded samples against the custom arrays demonstrated that sample Mel_4 was seropositive, while samples Mel_3 and Mel_5 were seronegative. Unblinding of the results revealed that the seroreactivity for NY-ESO-1 on the arrays for these samples was consistent with the ELISA data.

Identifying Potential Biomarkers on ProtoArray® Human Protein Microarrays

To identify potential biomarkers, it is necessary to compare the data from arrays probed with sera from patients with disease with data from arrays probed with sera from healthy individuals. In Figure 4, a plot of the signals from an array probed with serum from a patient with melanoma versus an array probed with serum from a healthy donor exhibited little or no correlation (R^2 value of 0.35). The observed differences in the autoantibody profile between these two samples could be due to disease-specific factors or to other parameters such as age, sex, diet, and environmental stimuli. To confidently identify potential disease-specific biomarkers from the profiling of sera on ProtoArray® Protein Microarrays, the number and types of samples must be carefully considered, and appropriate statistical algorithms must be employed.

	CV	R^2	Pearson
Healthy	14.9%	0.95	0.97
Melanoma	13.4%	0.98	0.99

Table 1—Experimental reproducibility. ProtoArray® Human Protein Microarrays v1.0 were probed in duplicate with a 1:150 dilution of serum from a healthy patient and serum from a melanoma patient. Reproducibility was evaluated by calculating replicate coefficients of variation, replicate R^2 values, and Pearson's Correlation Coefficients for all human protein features.

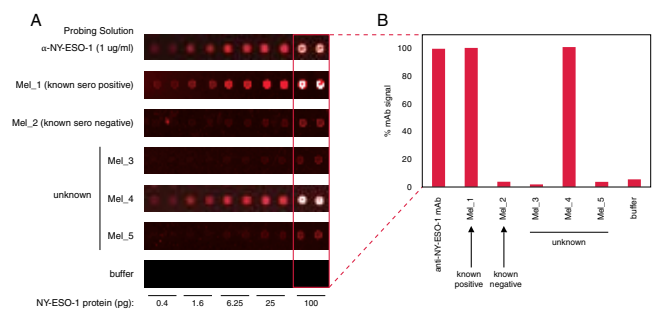


Figure 3—NY-ESO-1 reactivity of blinded serum samples. Custom protein arrays containing a gradient of NY-ESO-1 antigen were probed with 5 sera samples (Mel_1 to Mel_5), two characterized for seroreactivity (Mel_1 seropositive, Mel_2 seronegative) and three (Mel_3, Mel_4, Mel_5) characterized for seroreactivity but blinded during the study. Images and quantitation of signals are shown for each serum sample.

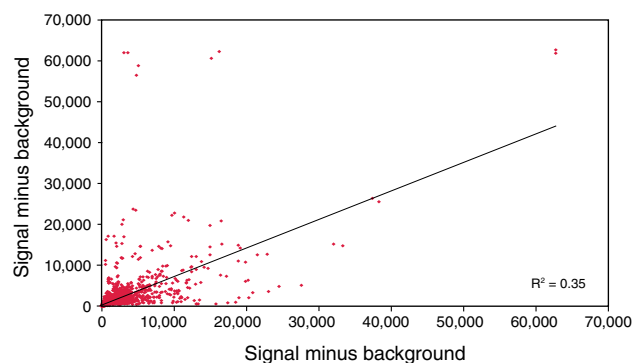


Figure 4—Identification of potential protein biomarkers. The signals from arrays probed with serum from a healthy individual (x-axis) and serum from a patient with melanoma (y-axis) were plotted. Note the lack of correlation for the two slides ($R^2 = 0.35$).

We next conducted a larger study in which serum samples from healthy donors ($n = 20$), melanoma patients ($n = 20$), and lung cancer patients ($n = 10$) were profiled on the ProtoArray® Human Protein Microarray v3.0, composed of approximately 5,000 human proteins, to identify melanoma-specific markers (Figure 1). Interestingly, a number of known melanoma autoantigens were observed in this study, including MAGEA10 and MAGEA12, consistent with data obtained by ELISA (data not shown) (17). However, the majority of these were observed in both melanoma and lung cancer samples, providing only general cancer markers rather than markers specific to a certain type of cancer. Importantly, our study identified a number of candidate biomarkers (>25) that were capable of differentiating the melanoma patients from both healthy individuals and lung cancer patients with a significant p-value (<0.05). An example of a novel, melanoma-specific biomarker candidate identified in this study is shown in Figure 5.

Summary

We have demonstrated the utility of ProtoArray® Human Protein Microarrays for profiling human sera to identify protein biomarkers for disease. The immune response profiling assay employed is highly sensitive and reproducible, and generates data consistent with other commonly utilized methods such as ELISA for biomarker identification. Most importantly, we present evidence indicating that high-content ProtoArray® Human Protein Microarrays from Invitrogen can empower investigators with the ability to uncover potentially novel protein biomarkers at an unprecedented pace. Panels of biomarkers discovered through ProtoArray® immune response profiling studies are expected to facilitate earlier diagnosis and more effective treatment of numerous human diseases, leading to a critical paradigm shift towards personalized medicine.

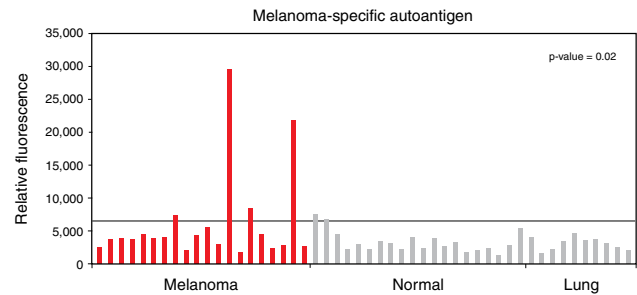


Figure 5—Candidate melanoma-specific biomarker. A p-value was calculated for each ProtoArray® Human Protein Microarray v3.0 feature, representing the probability that the differential distribution of signals observed across two populations would occur randomly. Candidate biomarkers were sorted, and those with significant p-values were evaluated for potential biological significance. The quantile-normalized, background-subtracted signals for a candidate melanoma-specific biomarker are shown, along with the associated p-value.

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