Quantitative Serum Proteomics from Surface Plasmon Resonance Imaging

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The detection and quantification of specific proteins in complex mixtures is a major challenge for proteomics. For example, the development of disease-related biomarker panels will require fast and efficient methods for obtaining multiparameter protein profiles. We established a high throughput, label-free method for analyzing serum using surface plasmon resonance imaging of antibody microarrays. Microarrays were fabricated using standard pin spotting on bare gold substrates, and samples were applied for binding analysis using a camera-based surface plasmon resonance system. We validated the system by measuring the concentrations of four serum proteins using part of a 792-feature microarray. Transferrin concentrations were measured to be 2.1 mg/ml in human serum and 1.2 mg/ml in murine serum, which closely matched ELISA determinations of 2.6 and 1.2 mg/ml, respectively. In agreement with expected values, human and mouse albumin levels were measured to be 24.3 and 23.6 mg/ml, respectively. The lower limits of detection for the four measurements ranged from 14 to 58 ng/ml or 175 to 755 pm. Where purified target proteins are not available for calibration, the microarrays can be used for relative protein quantification. We used the antibody microarray to compare the serum protein profiles from three liver cancer patients and three non-liver cancer patients. Hierarchical clustering of the serum protein levels clearly distinguished two distinct profiles. Thirty-nine significant protein changes were detected (p < 0.05), 10 of which have been observed previously in serum. α-Fetoprotein, a known liver cancer marker, was observed to increase. These results demonstrate the feasibility of this high throughput approach for both absolute and relative protein expression profiling. Molecular & Cellular Proteomics 7:2464–2474, 2008.

The use of antibody microarrays for proteomics analysis has grown steadily in the last few years as researchers look for high throughput measurements to complement their transcriptomics capabilities. First introduced for the detection of proteins and peptides (1, 2), antibody microarrays are now regularly used for analyzing samples from tissue and serum (3–7). Most protein-profiling microarray methods require fluorescence labeling of the samples (8, 9) often via secondary antibodies (10). Although these methods offer good specificity and sensitivity, they are often quite expensive and labor-intensive. Sandwich assays are difficult to develop as they require two specific antibodies per target protein. Labeling kits add additional cost and error to the measurement.

SPR imaging monitors protein-binding microarrays in a manner that is label-free, real time, and reusable. Because it is label-free, the process is fast, economical, and free of labeling biases. The real time aspect allows for binding kinetics to be observed and for the quality of the antibody-protein interaction to be evaluated. And as the arrays are reusable, a large number of samples can be processed on the same sensor chip efficiently.

In general, SPR techniques detect the changes in the effective refractive index (the thickness of the adsorbed layer) near the SPR-active surface. When polarized, collimated light is reflected from the active surface at an angle greater than the critical angle, and incident photons are coupled into surface plasmons, leading to a decrease in the reflected light intensity. Binding events at predefined positions increase the local refractive index, changing the reflectance pattern across the surface. This pattern is recorded in real time using a charge-coupled device camera (see Fig. 1.) Protein concentrations in a sample solution are determined by monitoring the changing of the refractive index that is caused by interaction of capture molecules on the surface with the targeted proteins (11). The ProteomicProcessor™ (Plexera Bioscience, Bothell, WA) is a Kretschmann configuration SPR instrument that can monitor the binding kinetics on a 1350-feature microarray with good detection limits (0.3 ng of protein/cm²) and a time resolution of 1 s (12). The instrument images microarray slides with a refractive index of 1.73, a gold thickness of 47.5 nm, and dimensions of 25 × 76 × 1 mm. Compatible slides include the NanoCapture Gold™ (Plexera Bioscience) and SPRGold™ (1-1001, Gentel Biosciences, Madison, WI). The active area of the array is a 14 × 14-mm square enclosed with a 70-μl flow cell. Samples can be injected at flow rates of up to 10 μl/s.

SPRI has been used to monitor a wide range of molecular interactions including carbohydrate-protein, peptide-protein,
A thin film of gold. Antibody spots are arrayed on the gold. The chip sensing chip consists of high index glass coated on the top side with that spot.

creases the refractive index and changes the fraction of light reflected from healthy, liver cancer, and other cancer subjects, and 3) antibodies to liver-specific proteins, 2) human serum samples.

tion of binding kinetics. Affinities were calculated using the kinetic constants and compared with the measurements of conventional immunoassays. The goal of this part of the study was to demonstrate that once proteins have displayed an interesting relative signal profile they can be further quantified in absolute terms.

EXPERIMENTAL PROCEDURES

Antibodies and Antigens—Antibodies were obtained from a number of sources. Chicken anti-transferrin (15-288-200, Hu31) was purchased from GenWay Biotech (San Diego, CA), goat anti-transferrin (840-128A, HuECT) was from Bethyl Laboratories (Montgomery, TX), and anti-albumin (GAL-90A, Hu45) was from Immunology Consultants Laboratory (Newberg, OR). Other liver-specific protein targets were selected based on transcriptomics analysis of 21 tissue types. Briefly the tissues were analyzed by the Solexa (Hayward, CA) massively parallel signature sequencing (MPSS) system, and transcripts were selected that were uniquely expressed in liver hepatocytes. The criteria used required that the transcripts be expressed in liver at 5 times the level of any other single tissue or 2 times the level of all other tissues combined. Additionally the expression level must exceed 10 transcripts/million, and orthologs must exist in both humans and mice. Of the 163 targets meeting the criteria, one or more antibodies were commercially available for 64, and 83 such antibodies were purchased. The antibody selection was not limited to targets detected previously in plasma or serum (21) of which there were 30. Additional, non-organ-specific monoclonal and polyclonal antibodies were provided by Drs. Alan Aderem, Qing Tian, Biao Yang Lin, and Hyuntae Yoo (ISB).

Human transferrin (T90190), mouse apotransferrin (T0523), human albumin (A3782), and mouse albumin (A1056) were purchased from Sigma-Aldrich. Normal human serum (IMS-SerOS) and mouse serum (IMS-CS7BL5-SER) were purchased from Innovative Research (Southfield, MI). Human cancer sera, both liver and non-liver cancer samples, were obtained from Asterand (Detroit, MI). Liver cancer serum samples were designated H1 (1043801, hepatic duct adenocarcinoma), H2 (19417A6, HCC), and H3 (41403A1, bile duct adenocarcinoma). Non-liver cancer serum samples were designated C1 (218302A, pancreatic adenocarcinoma), C2 (20998A1, breast cancer), and C3 (41403A1, colon cancer).

Antibody Microarray Printing—Microarrays were fabricated by pin-spotting antibodies on NanoCapture Gold SPR-active slides. Antibodies were diluted to 0.5 mg/ml (where possible; otherwise 0.2

and protein-protein binding as well as surface enzyme kinetics (13–17). Serum screening is a relatively new application for SPRI. Ferritin antibodies have been used with non-imaging SPR to quantify that tumor biomarker in human serum at levels as low as a nanogram per milliliter by Chou et al. (18).

Arrays of antibodies have been used with SPR to profile protein expression in mouse tissue with good reproducibility by Usui-Aoki et al. (19). Most recently, arrays of glycans have been used to detect infection by monitoring glycan-specific serum antibodies (20).

Serum provides a challenging test in complex protein mixtures in that there are potentially millions of different proteins spanning a dynamic range of many orders of magnitude with the most abundant 21 proteins constituting about 99% of the protein mass (21). Hence antibody-based detection methods face an enormous challenge in cross-reactivities.

The objective of this study was to determine whether SPR imaging provides quantitative proteomics data from a complex biological material such as serum. Methods were developed to print antibody microarrays and use them for determining either relative or absolute concentrations of proteins in serum. First, a high density antibody microarray containing a wide assortment of antibodies was printed, and protocols were tested to ensure reproducible binding and regeneration. Second, the microarray was used to compare the relative proteomic profiles of human serum from the healthy and cancer states. Third, the microarray was used to measure absolute protein concentrations in serum via the determination of binding kinetics.

Liver cancer serum was chosen for relative profiling because of the availability of the following: 1) a large number of antibodies to liver-specific proteins, 2) human serum samples from healthy, liver cancer, and other cancer subjects, and 3) a good protein biomarker to serve as a positive control. α-Fetoprotein (AFP) is a protein normally produced by immature liver cells in the fetus. Levels fall below 10 ng/ml by 1 year of age. In adults, levels of about 500 ng/ml are only seen in hepatic cell carcinoma (HCC), metastatic liver cancer, and germ cell tumors (22). The diagnostic sensitivity is about 60%. Additionally a number of studies have performed proteomics analysis of HCC serum and observed changes in specific protein levels. The goals of this part of the study were to determine whether high density SPR arrays can be used to discriminate between cancer states and if the individual protein changes are consistent with previously reported trends.

The serum proteins albumin and transferrin, from both human and mouse, were chosen for detailed quantitative analysis. Solutions of purified protein and serum were applied to the microarrays over a wide range of concentrations, and the binding kinetics were determined. Affinities were calculated using the kinetic constants and compared with the measurements of conventional immunoassays. The goal of this part of the study was to demonstrate that once proteins have displayed an interesting relative signal profile they can be further quantified in absolute terms.
mg/ml) in PBS and transferred to a 384-well microtiter plate. The antibody plate and the slides were loaded into a VersArrayTM robotic microarray spotter (Bio-Rad) configured to use two SMP7 contact pins (Telechem International, Sunnyvale, CA) that produce a 300-µm feature. The relative humidity was set to 60%. Each antibody was printed in duplicate. The printing resulted in arrays containing 22 rows × 36 columns of antibody features, including eight null features. After the arrays were incubated overnight in 70% relative humidity at 4 °C, they were washed with 10× PBS for 15 min with agitation, 1 min in 1× PBS, 1 min in 0.1× PBS, and 1 min in deionized water. The arrays were dried under a stream of nitrogen and placed in a nitrogen-filled box at 4 °C for storage. One day prior to use, the array surface was blocked with milk protein. The array was placed in a solution of 2% dry milk in PBS and allowed to incubate overnight at 4 °C. The array was then washed with 1× PBS for 60 min with agitation, 1 min in 0.1× PBS, and 1 min in deionized water and blown dry under a stream of nitrogen.

SPR Imaging Analysis—The antibody array was affixed with a 70-µl adhesive flow cell and placed in the SPR imager. Imaging and measurement were performed at the angle corresponding to an average antibody spot reflectivity of 30%. The rate of data acquisition was set to 0.2 Hz. The instrument was controlled and the data was collected using SPRit software version 1.2.0. A square region of interest 16 pixels high × 16 pixels wide was defined for each of the 792 antibody spots. Six background regions of interest of 10 × 16 pixels were defined at the top, middle, and bottom areas of the array. A simple two-point calibration was obtained by injecting 1× PBS (n = 1.3347 RIU) and 2× PBS (n = 1.4465 RIU) into the flow cell and recording the signal intensity at each point. The typical calibration factor was 2 camera units/microrefractive index unit (µRIU). All experiments were performed at 20 °C.

PBS (137 mM NaCl, 10 mM phosphate, 2.7 mM KCl, pH 7.4) was the SPR running buffer, 0.2% fish gelatin (G7765) from Sigma-Aldrich in PBS was the reblocking solution, and 3 mM HCl in deionized water was the regeneration solution. All solutions were degassed under a 50-kilopascal vacuum for 30 min. Each sample measurement cycle had four steps: reblocking, sample binding, array washing, and regeneration. Reblocking consisted of flowing 200 µl of reblocking solution through the flow cell at 2 µl/s. Binding occurred with the injection of 200 µl of sample followed by oscillatory flow (50 µl at 2 µl/s) for 5 min. The arrays were washed for 5 min with a constant flow of PBS at 2 µl/s. The array surface was regenerated by injecting 300 µl of regeneration solution, pausing flow for 3 min, and washing with 400 µl at 10 µl/s. An image of an antibody array appears in Fig. 2.

SPR Data Processing—Software was written by the Hood laboratory to process the large amount of raw binding data produced by the real time imaging (SPRISB version 1.0, available upon request). The software annotates the antibody array features, converts camera units to refractive index units, and performs background referencing to remove bulk refractive index effects. The software allows the measurement of binding signal responses at various time points in units of refractive index and exports the data to formats usable by spreadsheets, TIGR MeV and CLAMP XP (23). Expression profiles were plotted as heat maps using MeV.

Sample Injection and Surface Regeneration—Binding and regeneration protocols have been well studied in non-imaging SPR applications (24). Binding buffers are usually based on PBS or HEPES often with a carrier protein and a small amount of non-denaturing detergent. The carrier protein may serve to coat the walls of the fluidic system and flow cell, minimizing the loss of the protein analytes to adsorption. Detergents can also help minimize adsorption and non-specific binding. Regeneration solutions can be based on acid, base, salt, or detergent. An acid such as HCl or H3PO4 in the range of 1–100 mM is common.

An SPRI microarray was subject to repeated injections of mouse transferrin to test the efficacy of the surface regeneration. Thirteen cycles of reuse consisted of sample injection, washing, and 3 mM HCl regeneration. One of three cycles used a sample of 5 µg/ml transferrin in PBS. The other two cycles were blank injections (PBS only).

Serum Protein Profiling—The seven human serum samples were diluted 50-fold in PBS. The samples were applied serially to the same microarray in the order H1, C1, H2, C2, control, H3, and C3. Responses after 5 min of binding and 5 min of washing for each antibody were recorded. Each array contains two replicate antibodies; these were averaged. Binding signals ranged from approximately ~50 to 2550 µRIU. A signal floor was set at 25 µRIU. Features that did not show at least 75 µRIU of binding during any of the seven injections were discarded. Signals were normalized by calculating the logarithmic ratios (base 2) of the cancer signals to the control signals. These ratios were imported into TIGR MeV for automated analysis. Hierarchical clustering was performed for the antibodies (“genes” in MeV terminology) and the sera (“experiments” in MeV terminology) using the average linkage method. The significantly differential proteomic features were determined by the MeV t test (p < 0.05).

Serum Protein Quantification—The microarrays were calibrated using a range of known concentrations of pure proteins. After calibration, the microarrays were used to analyze a series of serum dilutions. Three proteins were injected in concentrations of 156, 625, 2500, and 10,000 ng/ml. Mouse transferrin was injected in concentrations of 50,
400, 2000, and 10,000 ng/ml. Human serum was diluted by factors of 1600, 400, 100, and 25 and injected in that order. Mouse serum was diluted by factors of 3200, 800, 200, and 50 and injected in that order. Each series of injections was preceded by a null injection (a blank sample containing only PBS buffer).

The SPR curve-fitting software package CLAMP XP was used to solve for kinetic parameters and the serum protein concentrations. First a reaction scheme was specified on the modeling page. As the antibodies of interest are polyclonal and as they are immobilized on the gold surface by random orientation, a heterogenous ligand model was chosen (25, 26). This model assumes the antibody-antigen interaction is composed of two independent types of binding events. At low concentrations, a higher affinity interaction, component 1, dominates. At high concentrations, both component 1 and component 2 contribute measurably to the signal.

CLAMP was configured with a two-component reaction scheme. Component 1 is “A + B ⇋ AB,” and component 2 is “A + B ⇋ AC” where A is the antigen, B is the favorably oriented capture antibody component, and C is the unfavorably oriented capture antibody component. Curve fitting proceeded in two steps. First, two sets of on-rates (k_{on}), off-rates (k_{off}), and binding responses were determined (six parameters). The binding data from the calibration protein were loaded, and the six parameters were determined iteratively. To ensure that the iterative solver converged on a solution, the off-rates were constrained to values ≥ 10^{-4} s. As the washing process was limited to 5 min, it is impossible to accurately observe off-rates slower than 10^{-4} s. Second, the serum binding data were loaded, and a seventh unknown parameter, serum stock concentration, was added to the model. Using the previously determined six parameters as starting values, the iterative solver was used to simultaneously fit the seven parameters.

A commercially available human transferrin ELISA kit (Bethyl Laboratories E80-128) was used to quantitatively analyze the human serum according to the manufacturer’s instructions. Protein concentrations were reported as μg/ml based on the standard curve.

RESULTS

Regeneration—Regeneration of the microarray surface was highly successful. A weak HCl solution was found to completely strip serum proteins from the arrayed antibodies without damaging the antibodies. Fig. 3 shows that transferrin kinetics and binding levels are unchanged after 12 regeneration cycles.

Serum Protein Profiling of Liver Cancer—The binding signals (the increase in signal after binding and washing) for the 384 pairs of features ranged from -80 to 4900 μRIU and followed a Poisson distribution with a λ (average and S.D.) of 86 μRIU. The 12 null features (where spotting buffer containing no antibody was printed) produced signals of 24 ± 14 μRIU. On average, only 27 antibody features exhibited binding signals of less than 10 μRIU. For calculating logarithmic ratios, these signals were adjusted to a floor level of 25 μRIU.

Hierarchical clustering divided the serum samples into two distinct groups: liver cancer and non-liver cancer. Of the 384 different microarray features, 259 antibodies produced detectable binding signals (greater than 75 μRIU) in at least one serum sample. The clustering of these features is shown in Fig. 4. Of these, 174 produced detectable signal in all seven samples.

Thirty-nine proteomic features were found to differ significantly between the two groups using the MeV t test at the α = 0.05 level. Thirty-five were increased, and four were decreased (see Table I). The AFP level was found to increase from 59 ± 6 μRIU in the non-liver cancer group and 63 μRIU in the control to 82 ± 7 μRIU in the liver cancer group. In addition to AFP, the protein targets of eight other antibody features have been reported previously to increase in the serum of liver cancer patients. These are dipeptidyl-peptidase 4 (27), complement C4 (28), tumor necrosis factor (29, 30), interleukin 4 (30), interleukin 6 (29, 31), interleukin 10 (31), catalase, transferrin, and carbamoyl-phosphate synthetase 1 (32). Similarly, fructose-bisphosphate aldolase has been observed previously (33) to decrease in the serum of HCC patients. Two-thirds of the targets of these features (26 of 39) also correspond to genes observed previously to show differential mRNA regulation in HCC tissue (32, 35, 36). For example, Xu et al. (36) compared HCC and noncancerous liver cells
on a 12,000-gene array and found 19 of the targets listed in Table I to be differentially expressed.

**Serum Protein Quantification**—The two-component model was found to closely fit the observed binding behavior. In all four cases, the two-component model was found to reduce the sum-squared error by at least a factor of 2. Observed and fitted response curves appear in Fig. 5, and the parameters are summarized in Table II. The transferrin level in human serum was found to be 2.0 mg/ml, which closely matched the 2.6 mg/ml obtained by ELISA analysis. The transferrin level in murine serum was found to be 1.2 mg/ml both by SPR and by ELISA analysis.

Also in agreement with expected values, human and mouse albumin levels were measured to be 24.3 and 23.6 mg/ml, respectively. These results are all consistent with the reference ranges of 30–50 mg/ml for albumin and 1–3 mg/ml for transferrin (37, 38). Component 1 on-rates ranged from $9.01 \times 10^4$ to $5.86 \times 10^5$ M$^{-1}$ s$^{-1}$, and off-rates were all less that $1.01 \times 10^{-3}$ s$^{-1}$. Because of the short washing time of 5 min, off-rates below $10^{-4}$ s$^{-1}$ cannot be precisely determined. Based on these kinetics, the component 1 affinities were all less than 1.72 nM. Component 2 on-rates ranged from $5.40 \times 10^3$ to $5.36 \times 10^5$ M$^{-1}$ s$^{-1}$, and off-rates ranged from $1.23 \times 10^{-2}$ to $4.29 \times 10^{-4}$ s$^{-1}$. Based on these kinetics, the component 2 affinities ranged from 23 to 125 nM.

**Sensitivity and Specificity**—The limits of detection for the four measurements were all in the picomolar range. The limit is defined as the concentration producing a binding signal after washing greater than that of the blank injection plus three times the root mean square noise. Human transferrin was detectable to 14 ng/ml, human albumin was detectable to 50 ng/ml, mouse transferrin was detectable to 58 ng/ml, and mouse albumin was detectable to 52 ng/ml. This corresponds to molar concentrations between 175 and 755 pM.

The level of cross-reaction (or unwanted nonspecific antibody binding) was found to be quite low for the four serum proteins. The affinity was determined for all SPR array antibodies reacting detectably with mouse and human forms of albumin and transferrin. For the nearly 400 different antibodies arrayed, only 19 instances of cross-reaction were observed (see Table III). In all these cases, the affinities were weaker than the specific binding affinities. Affinity was calculated from the kinetic rates assuming a simple, one-compartment model.

**Other Technical Observations**—Key factors in obtaining reproducible SPR sensorgrams included slide washing, mi-
croarray blocking, and buffer degassing. Slides that were washed in 1× PBS often exhibited poor morphology. Printed material could be streaked across the array by the washing, giving the appearance of "comet tails" from bright antibody spots. Slides that were washed in the blocking buffer had fewer comet tails but often exhibited a dirtier background. The 1× PBS buffer seemed to slow the binding of excess antibody to the background, resulting in the fewest comet tails and the cleanest background. Several blocking proteins were tried with varying results: 2% milk, 1% fish gelatin, and 1% casein all provided good blocking for microarrays exposed to only single protein solutions. The milk and casein blocking of the gold surface equilibrates after about 2 h. The fish gelatin equilibrates within 5 min. Significant background binding was observed (>50 μRIU) when serum was applied to fish gelatin- or casein-blocked slides. Milk proved to be the best blocker for serum samples. Additionally a quick reblock with fish gelatin appears to replace any milk protein lost after each regeneration cycle. The use of degassed solutions was also found to be very important in avoiding the appearance of small bubbles in or around the viewing area of the imaging camera. The bubbles could be detected by the appearance of a small sinusoidal waveform superimposed on the sensorgrams of nearby microarray features. Generally this artifact is eliminated by local background subtraction.

**DISCUSSION**

This study of serum samples found that accurate, absolute proteomics quantification could be obtained from a high

**TABLE I**

| Antibody target                                      | SPR log$_2$ change | t test | HCC serum literature | HCC-related |
|------------------------------------------------------|--------------------|--------|----------------------|-------------|
| **Ubiquitin**                                        | 0.821              | 0.002  |                      | Yes         |
| **Sodium/potassium-transporting ATPase β-2 chain**   | 0.989              | 0.005  |                      | Yes         |
| **T-cell surface antigen T3/Leu-4 ε chain**          | 0.639              | 0.009  |                      | Yes         |
| **Nanog**                                            | 0.934              | 0.009  |                      | Yes         |
| **Carbamoyl-phosphate synthetase 1**                 | 0.667              | 0.010  | Yes                  | Yes         |
| **Plasma retinol-binding protein precursor**         | 0.610              | 0.012  |                      | Yes         |
| **α-Fetoprotein (AFP)**                              | **0.465**          | **0.013** | Yes                  | Yes         |
| Adenylate kinase 2                                   | 0.643              | 0.013  |                      | Yes         |
| Death-associated protein kinase 3                    | 0.369              | 0.014  |                      | Yes         |
| β$_{18}$-Glycoprotein 3 hemopexin                     | 0.713              | 0.017  |                      | Yes         |
| Dipeptidyl-peptidease 4 or CD26                      | 1.772              | 0.018  | Yes                  | Yes         |
| Protein-tyrosine phosphatase, receptor-type, Z polypeptide 1 | -0.682             | 0.018  | Yes                  | Yes         |
| Monocyte chemoattractant protein 1                   | 0.407              | 0.018  |                      | Yes         |
| Chemokine ligand 5                                   | 1.318              | 0.019  |                      | Yes         |
| CD16/CD32                                            | 0.789              | 0.019  |                      | Yes         |
| Transferrin                                          | 0.398              | 0.021  | Yes                  | Yes         |
| Proto-oncogene tyrosine-protein kinase                | 0.835              | 0.024  |                      | Yes         |
| Interleukin-12                                       | 0.867              | 0.025  |                      | Yes         |
| Fructose-bisphosphate aldolase                       | -1.054             | 0.027  | Yes                  | Yes         |
| G protein-coupled receptor 38                        | 1.129              | 0.028  |                      | Yes         |
| CD16/CD32                                            | 0.543              | 0.028  |                      | Yes         |
| CD8a                                                 | 1.367              | 0.029  |                      | Yes         |
| CD36 antigen-like 2                                  | -0.913             | 0.031  |                      | Yes         |
| Renal carcinoma antigen NY-REN-30                    | 0.492              | 0.031  |                      | Yes         |
| Complement factor H                                  | -0.651             | 0.032  |                      | Yes         |
| Catalase                                             | 0.559              | 0.033  | Yes                  | Yes         |
| Complement C4                                        | 0.554              | 0.033  | Yes                  | Yes         |
| Aldehyde dehydrogenase 4A1                           | 0.574              | 0.035  |                      | Yes         |
| ATP2B1, plasma membrane calcium ATPase isofrom 1     | 0.510              | 0.036  |                      | Yes         |
| CD45                                                 | 0.732              | 0.038  |                      | Yes         |
| Tumor necrosis factor                               | 0.856              | 0.038  | Yes                  | Yes         |
| Tumor necrosis factor receptor superfamily member 5 | 1.033              | 0.038  |                      | Yes         |
| Hepatocyte-derived fibrinogen-related protein 1      | 0.412              | 0.038  |                      | Yes         |
| Interleukin-4                                        | 0.683              | 0.039  | Yes                  | Yes         |
| Tumor necrosis factor receptor superfamily, member 5 | 0.802              | 0.040  |                      | Yes         |
| Interleukin-10                                       | 0.667              | 0.041  | Yes                  | Yes         |
| Interleukin-6                                        | 0.794              | 0.043  | Yes                  | Yes         |
| Fibroblast growth factor 4                           | 0.722              | 0.047  |                      | Yes         |
| β-Catenin                                            | 3.198              | 0.049  |                      | Yes         |
throughput microarray system. Sample preparation was fast and convenient. Serum was simply diluted and injected into the microarray flow cell. The samples required no labeling, so the associated effort, expense, and artifacts were avoided. Label-free arrays also require only a single antibody, a useful property as often researchers do not have a compatible secondary antibody available for use in a sandwich assay. The microarray fabrication is also relatively simple as the antibodies were printed with a conventional DNA spotter using familiar protocols. Hundreds of microarrays can be printed with a 10-µL volume of antibody, and many samples can be analyzed with each array. Because the arrays can be regenerated, interarray variability can be eliminated, and more experimental replicates can be conducted. SPR microarrays also have the benefit of real time analysis.

Unlike the end point scanning of most microarray systems, real time detection provides a great deal of useful information about the probe-target interaction. First, the amount of antibody (or other capture molecule) initially present is visible. This provides a level of quality control before precious sample is consumed. Problems with microarray printing such as poor morphology or weak immobilization are immediately apparent. Second, the blocking step can be monitored to ensure it goes to completion. Third, the total protein concentration of a sample can be inferred from its bulk refractive index if desired. Fourth, signal saturation is detectable. A sudden plateau on the sensorgram of a feature indicates that all binding sites have been occupied. Last and most importantly, real time analysis allows the calculation of kinetic parameters. Kinetic parameters are useful for determining the affinity constant of an antibody and for absolute quantification on other microarrays. If kinetic parameters are unavailable, quantification requires a standard curve for each array because each varies in the amount of immobilized antibody. When kinetic parameters are known, concentration can be calculated from binding rates.

The results of this study suggest that SPRI analysis of antibody microarrays is an efficient means of serum protein profiling. Differential protein abundances were observed between HCC and non-HCC serum samples including a number of previously reported examples. The novel observations could be sample collection artifacts, statistical anomalies, or potential biomarkers. A general biomarker screening strategy would involve first obtaining relative protein concentrations from a series of samples bound to the same array. Relative protein levels may be inferred from binding signals when the same microarray is used for all samples and provided that the surface regeneration is complete. If the regeneration protocol is too aggressive, antibody can be removed from the surface. If the protocol is too mild, antigen can remain bound. In either case, the binding signals will tend to decline over time. This potential problem can be checked by repeating the experiment multiple times and reversing or randomizing the order of sample injections. Once potential biomarkers emerge, those proteins can be purified and used to obtain antibody-antigen kinetics. The kinetics could then be used to calculate the absolute protein concentrations in the original samples.

SPRI detection of antibody microarrays provides very different advantages and disadvantages than label-based detection methods like fluorescence. The major advantages are
the simplicity of sample preparation, array reusability, and real time readout. The real time readout provides kinetic data that assist in protocol optimization, the study of low affinity interactions, and absolute quantification. On the other hand, sensitivity, specificity, and dynamic range parameters can all be limiting in cases.

Sensitivity is a concern for biomarker screening as many serum proteins, such as the interleukins, are normally found in the pg/ml range, whereas the limits of the system here are on the order of 25 ng/ml. A number of cytokine ELISAs using chemiluminescence detection can detect 1 pg/ml (39). Several fluorescence label protein microarrays report detection limits in the very low ng/ml range (2, 40). This sensitivity can be modulated by adjusting the number of labels per molecule. The magnitude of a signal in an SPR assay is a function of the molecular weight and antibody affinity as well as the sample concentration. Where greater sensitivity is required, the mass provided by a secondary antibody may be used to amplify the signal (41). Even without amplification, many disease-related serum proteins normally occur at measurable levels. Potential tumor markers such as epidermal growth factor receptor (42), IGFBP-2 (43), TIMP-1 (44), matrix metalloproteinase-9 (45), osteopontin (46), pepsinogen I (47), and RANTES (regulated on activation normal T cell expressed and secreted) (48) are all found at concentrations above 25 ng/ml in control subjects. Additionally the well established cancer biomarkers prostate-specific antigen (49), carcinoembryonic antigen (50), and α-fetoprotein (34) are often elevated well above that level in the disease state.

A second important concern for proteomics applications will be the challenge of “off-target” binding. Single antibody assays require highly specific binding properties, whereas a sandwich assay can use two less specific antibodies that, in combination, provide sufficient specificity. Even the best antibodies can exhibit cross-reactivity with off-target antigens. Although the four antibody-antigen interactions studied here turned up a relatively low number of examples, this was aided by two very favorable conditions. First, proteins such as albumins and transferrins have a large number of antibodies commercially available. Those displaying low specificity have likely been discarded by the marketplace. There are far fewer antibody choices for obscure protein targets, so average specificity will likely be lower. Second, these proteins are more abundant in serum than their cross-reacting competitors. When the ratios of target/off-target proteins are reversed, even a low affinity cross-reaction could dominate the SPR signal. Antibody microarrays remain limited by the quality of the binding reagents available. As antibody development improves so will the quality of antibody array analysis.

The dynamic range of SPRI is a third constraint as it is limited by the width of the linear region of the surface plasmon resonance curve and the data resolution (bits) of its camera detector. Because the dynamic range of the serum proteome is very large, serum protein profiling will usually require sam-
The affinity was determined for all SPR array antibodies reacting detectably with mouse and human forms of albumin and transferrin. For the nearly 400 different antibodies arrayed, only 19 instances of off-target binding were observed. In all cases, the fastest on-rates were exhibited by the specifically targeted antigens. Affinity was calculated from the kinetic rates assuming a simple, one-compartment model. Hum, the nearly 400 different antibodies arrayed, only 19 instances of off-target binding were observed. In all cases, the fastest on-rates were exhibited by the specifically targeted antigens. Affinity was calculated from the kinetic rates assuming a simple, one-compartment model.

## Table III

**Serum protein cross-reactivity**

The affinity was determined for all SPR array antibodies reacting detectably with mouse and human forms of albumin and transferrin. For the nearly 400 different antibodies arrayed, only 19 instances of off-target binding were observed. In all cases, the fastest on-rates were exhibited by the specifically targeted antigens. Affinity was calculated from the kinetic rates assuming a simple, one-compartment model. Hum, the nearly 400 different antibodies arrayed, only 19 instances of off-target binding were observed. In all cases, the fastest on-rates were exhibited by the specifically targeted antigens. Affinity was calculated from the kinetic rates assuming a simple, one-compartment model.

| Analyte name | Antibody target | Code | For | From | Type | $k_{off}$ | $k_{on}$ | Affinity | Response |
|--------------|----------------|------|-----|------|------|----------|----------|-----------|----------|
| Mouse albumin | Albumin | Hu45 | Mouse | Rabbit | Poly | 610,000 | 7.92 × 10^{-4} | 1.30 | 48.1 |
| Mouse albumin | Albumin | AAC | Hum | Goat | Mono | 17,500 | 9.73 × 10^{-4} | 55.7 | 51.4 |
| Mouse albumin | Transferrin | Hu31 | Hum, mouse, rat | Chicken | Poly | 6,410 | 9.89 × 10^{-4} | 154 | 44.3 |
| Mouse albumin | C4 complement | H33 | Hum | Mouse | Poly | 667 | 1.18 × 10^{-2} | 17,700 | >600 |
| Mouse albumin | Transferrin | Hu43 | Hum, mouse, rat | Goat | Mono | 259 | 2.21 × 10^{-3} | 8,530 | 526 |
| Mouse transferrin | Transferrin | Hu43 | Hum | Goat | Mono | 123,000 | 2.19 × 10^{-3} | 17.9 | 64.0 |
| Mouse transferrin | Transferrin | Hu31 | Hum, mouse, rat | Chicken | Poly | 98,000 | 1.80 × 10^{-3} | 18.4 | 165 |
| Mouse transferrin | Apolipoprotein B | Hu29 | Hum, mouse | Rabbit | Poly | 65,400 | 1.00 × 10^{-3} | 15.3 | 21.9 |
| Mouse transferrin | Transferrin | ATC | Hum | Goat | Mono | 64,600 | 2.76 × 10^{-3} | 42.6 | 43.9 |
| Mouse transferrin | Transferrin | Hu81 | Hum | Mouse | Mono | 35,700 | 6.59 × 10^{-4} | 18.5 | 36.6 |
| Human albumin | Albumin | Hu45 | Mouse | Rabbit | Poly | 386,000 | 1.08 × 10^{-3} | 2.80 | 25.3 |
| Human albumin | Albumin | AAC | Hum | Goat | Mono | 74,600 | 3.02 × 10^{-4} | 4.05 | 189 |
| Human albumin | ASGPR | Hu30 | Rat | Mouse | Mono | 46,500 | 9.87 × 10^{-4} | 21.2 | 89.9 |
| Human albumin | Haptoglobin | Hu32 | Hum, mouse, rat | Chicken | Poly | 38,700 | <10^{-4} | 2.59 | 565 |
| Human albumin | Trypsin | Hu35 | Hum, mouse, rat | Chicken | Poly | 11,500 | <10^{-4} | 8.68 | 116 |
| Human albumin | Transferrin | Hu81 | Hum | Mouse | Mono | 8,570 | 2.67 × 10^{-4} | 31.2 | 167 |
| Human albumin | Transferrin | Hu31 | Hum, mouse, rat | Chicken | Poly | 5,830 | 1.61 × 10^{-3} | 275 | 57.2 |
| Human albumin | ASL | Hu51 | Hum | Mouse | Mono | 3,460 | 1.01 × 10^{-3} | 292 | 95.5 |
| Human albumin | Transferrin | ATD | Hum | Goat | Mono | 3,230 | 2.11 × 10^{-3} | 653 | 91.9 |
| Human transferrin | Transferrin | ATC | Hum | Goat | Mono | 278,000 | 5.59 × 10^{-4} | 2.01 | 62.2 |
| Human transferrin | Transferrin | Hu31 | Hum, mouse, rat | Chicken | Poly | 110,000 | 1.44 × 10^{-4} | 1.30 | 357 |
| Human transferrin | Transferrin | ATD | Hum | Goat | Mono | 94,300 | 1.10 × 10^{-3} | 11.7 | 114 |
| Human albumin | Albumin | AAC | Hum | Goat | Mono | 61,700 | 2.54 × 10^{-4} | 4.11 | 209 |
| Human transferrin | Albumin | AAD | Hum | Goat | Mono | 53,600 | 1.92 × 10^{-3} | 35.7 | 89.3 |
| Human transferrin | Trypsin | Hu35 | Hum, mouse, rat | Chicken | Poly | 36,500 | 5.71 × 10^{-4} | 15.6 | 112 |
| Human transferrin | Ferritin | Hu34 | Hum | Mouse | Mono | 35,900 | 2.01 × 10^{-3} | 56.0 | 29.2 |
| Human transferrin | Haptoglobin | Hu32 | Hum, mouse, rat | Chicken | Poly | 31,900 | 6.37 × 10^{-5} | 2.00 | 353 |
| Human transferrin | CD16/32 | CD16 | Hum | Mouse | Mono | 22,500 | 2.15 × 10^{-3} | 95.5 | 40.6 |
| Human transferrin | C3 complement | ACC | Hum | Chicken | Poly | 21,600 | 3.12 × 10^{-4} | 14.5 | 227 |
| Human transferrin | C3 complement | Hu27 | Hum, mouse, rat | Sheep | Poly | 20,000 | 5.31 × 10^{-4} | 26.6 | 33.4 |

It is also conceivable that signals produced by unintended cross-reactions might also be diagnostically useful if they display the dynamic envelope of phenotypic responses. In some cases it may be possible to find a single antibody measurement that constitutes a sensitive and specific biomarkers; in other cases, it may require sets of antibody measurements or panels of protein biomarkers to produce diagnostic information.

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