S&S® Serum Biomarker Chip Displays Specificity & Reproducibility for 120 Different Human Biomarker Profiles

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Abstract

We introduce a unique tool for the determination of relative abundances for human serum biomarkers. Conceptually similar to DNA microarrays, the S&S® Serum Biomarker Chip (SBC) is a single capture antibody array that was developed for comparative analysis of serum samples in order to identify differences or similarities in protein expression profiles. The SBC array is manufactured on a nitrocellulose two-pad FAST® slide in which each pad contains 120 antibodies specific for circulating tumor-related proteins printed in triplicate and arranged as four quadrants per pad. The Universal Linkage System (ULS™) protein labeling kit was employed to label serum proteins with either biotin or fluorescein prior to incubation with the SBC array. Data from the array was collected using an Axon scanner to detect fluorescent emitted from Dyomics dyes DY™647 and DY™547 conjugated to Streptavidin and anti-Fluorescein antibody respectively. We show that fluorescently labeled biomarker proteins bind specifically to their capture antibody as determined via competition experiments. The system was tested for reproducibility by running eleven individual experiments and measuring the variation in the signal intensity of each target biomarker. The results show that the CV is less than 20% for 105 of the 120 capture biomarker antibodies. We evaluated array sensitivity by spiking FCS with a cocktail consisting of 26 different biomarker antigens at concentrations of 500, 100, and 25 ng/ml. In addition, we were able to observe differences in the labeling patterns when serum samples from individuals with breast, colon, prostate and bladder cancer were compared to age and gender matched controls. The SBC array, when coupled with the ULS protein labeling system, provides the research community with a powerful tool to analyze the relative abundances of 120 individual biomarkers in human serum, requiring significantly less time and money as compared to ELISA techniques.

Introduction

The S&S Serum Biomarker Chip is designed to study relative protein abundance in human serum samples. This product addresses a growing need for technologies that enable broad molecular profiling of biological samples. The ability to identify multiple serum biomarkers simultaneously has many applications in basic biological research. The use of DNA arrays for profiling mRNA expression in cells has provided valuable information in many biological areas. However, since there is not always a direct correlation between the mRNA level and the expression of the protein, a method that can assay multiple proteins is required for a meaningful analysis. Antibody chips provide such a solution and can be used to profile abundance of proteins in samples. The S&S Serum Biomarker Chip contains 120 different antibodies representing serum biomarkers associated with human disease states of every major organ. The product enables scientists to reproducibly pattern the relative abundance of 120 human serum proteins between two samples, such as serum samples from diseased and healthy individuals. The Serum Biomarker Chip is not intended to provide quantitation such as an ELISA method would provide, but rather is a method to monitor or discover protein abundance changes between biological samples on a broad scale.

The S&S Serum Biomarker Chip uses antibody microspots to capture hapten-labeled serum proteins followed by readout using fluorescent reporter molecules. The entire procedure, from sample labeling to slide scanning, takes one 24 hour day to complete since we recommend an overnight incubation. It is intended to be used with human serum proteins labeled with small molecular weight haptens. The hapten swapping manipulations used in this were necessary to control for variations in labeling efficiency. A hapten swapping experiment accounts for hapten-specific differences in either Biotin-ULS™ or Fluorescein-ULS™ labeling efficiencies or differences in antibody-antigen binding interactions caused either by steric hindrance or solubility issues associated with chemically labeled serum proteins. Here, each pad on the slide is probed with two different serum samples labeled with different haptens, and the second pad is probed with the haptens reversed. The normalized intensity for each element of each pad is calculated as the average of the biotin- and fluorescein-labeled derived intensities from a two-pad experiment. This method is attractive for antibody chips as it takes into account any hapten-specific differences in antigen-antibody interactions.

Methods & Results

Table 1. The chart below shows a list of the antibody specificity targets present on the Serum Biomarker Chip. Biomarker-specific antibodies were printed on a two-pad S&S FAST® Slide using a Cartesian ProSys 5510 arrayer with SMP3XB pins (TeleChem International, Inc.). Each pad consists of an identical array and each array is comprised of four subarrays. The antibodies were arrayed in triplicate in a proprietary array buffer with approximately 1ng of antibody deposited per spot. The antibodies were spotted at a pitch of 0.5mm.

Alpha1 antichymotrypsin	Alpha fetoprotein	Angiogenin	Alpha2 macroglobulin
Apolipoprotein	Angiostatin	Bone sialoprotein	Angiopoietin-2
Beta2 microglobulin	CA125	Cathepsin B	Apolipoprotein J
CA15-3	CA50	Ceruloplasmin	Chorionic gonadotropin-beta
CA19-9	Carcinoembryoinc antigen (gp 2)	C-reactive protein	Epidermal growth factor receptor
Carcinoembryonic antigen (gp 4)	Chorionic gonadotropin-alpha	Cyclin-dependent kinase inhibitor 2A	Fibroblast Growth factor-basic
Chondroitin Sulftate	Cytokeratin Fragment 21-1	E-Selectin	Hepatocyte Growth Factor
Chromogranin	Fas ligand	Estrogen Receptor	IgG
Collagen Type I	Haptoglobulin	FAS	IL-13
Complement C4	IgA	Ferritin	IL-17
Eotaxin	IgG	Fibroblast Growth factor-7	IL-2 receptor-alpha
Epidermal growth factor	IgM	GM-CSF	IL-8
ErbB2	IL-12p40	IgG	Insulin growth factor binding protein 3
G-CSF	IL-1-alpha	IL-10	Interferon-gamma
Hemoglobin	IL1-beta	IL-2	Kallikrein-9
ICAM-1	IL-3	Insulin-like growth factor 1	Myeloperoxidase
IgG	IL-4	IP-10	Neuron Specific Enolase
IL12-p70	IL-6	Laminin	PDGF (all isoforms)
IL-2 receptor-beta	Insulin	MCP-1	PDGF (BB isoform only)
IL-5	Kallikrein-14	MCP-2	Placental Alkaline Phosphatase
IL-7	Kallikrein-5	M-CSF	plasminogen activator inhibitor
Kallikrein-12	Low-density lipoprotein	MIP-1-alpha	Prostatic acid phosphatase
MCP-3	MCP-4	Myoglobin	S100
MMP-3	MMP-2	PSA (Free)	Serum Albumin
Plasminogen	MMP-9	PSA-Alpha-chemotrypsin complex	Tetranectin
RANTES	Osteopontin	Serum Albumin	Thrombopoietin
Serum Albumin	PSA (Total)	TGF-alpha	Thrombospondin-1
TAG-72	Serum Albumin	Tyrosinase	TNF-alpha
TGF-beta	Sialyl Lewis X	Urokinase Plasminogen Activator	Tumor-Associated Trypsin Inhibitor
Thyroglobulin	TIMP1	VCAM-1	VEGF-D
TIMP2	TNF-beta	VE-Cadherin	Von Willebrand Factor
VEGF	Transferrin		

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Figure 1. Direct labeling of human serum proteins using the Universal Labeling System - detection of Biomarkers spiked into Human Serum. The graph demonstrates that the ULS protein labeling reagent successfully labels exogenous proteins "spiked" into human serum. The ULS labeling system reacts specifically with the free sulfhydryl group of methionine. Two proteins were used for this spiking experiment, epidermal growth factor (EGF) and prostate specific antigen (PSA). EGF contains a single methionine, while PSA contains two methionine residues. As shown, both EGF and PSA were spiked into serum obtained from a healthy individual yielding a final concentration of 10ug/ml. One microliter of this spiked serum was labeled using the ULS-biotin system in a 50 ul reaction volume (final concentration = 200 ng/ml). The reaction was processed to remove un-reacted ULS-biotin and subsequently incubated on a FAST slide spotted with the appropriate capture antibodies. The slide was washed and incubated with streptavidin conjugated to Cy3. Detection of EGF and PSA is shown in the graph below.

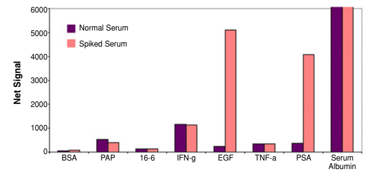
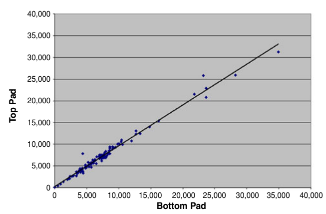


Figure 2. SYPRO® Ruby staining of the S&S Serum Biomarker Chip. Serum Biomarker Chips were treated with the protein stain SYPRO® Ruby according to the manufacturers instructions. The average signal intensity for each antibody triplicate on both the top and bottom pad were plotted and the regression line drawn (R^2=0.98). The slide was scanned with the Axon 4200A scanner using the blue laser at λ488 and a PMT setting of 300 and laser power of 95%. Data analysis was completed by using Array Vision FAST Software.

Printing Reproducibility of the array compares both pads of the slide: Total protein staining by SYPRO® Ruby demonstrates that nearly identical quantities of antibody are printed on both the top and bottom pad of each two pad S&S Serum Biomarker Chip.



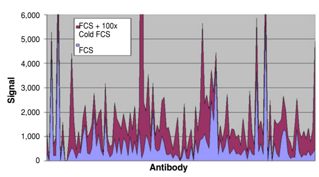
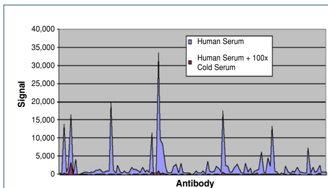


Figure 3. Specificity of the capture antibodies for Human Serum Biomarkers:
a.) Fetal Calf Serum: Four microliters of fetal calf serum (FCS) was labeled for 7 hours with ULS-biotin. These labeled proteins were incubated for 16 hours on the Serum Biomarker Chip either in the presence or absence of a 100-fold excess of unlabeled ("cold") FCS. The results clearly show that the cold FCS failed to inhibit the binding of the labeled FCS proteins suggesting that much of the binding observed under these conditions was the result of non-specific interactions of bovine proteins with the anti-human antibodies.



3b. Human Serum: Four microliters of human sera was labeled with ULS-biotin for 7 hours. These labeled proteins were incubated for 16 hours on the Serum Biomarker Chip either in the presence or absence of a 100-fold excess of unlabeled ("cold") human serum. Unlabeled human serum inhibited nearly all the signal at all the spots on the array. This result demonstrates that the binding of human proteins to the Serum Biomarker chip is principally the result of specific binding of the antigens with their cognate antibodies.

Figure 4. Average signal intensity and %CV of protein expression ratios from multiple Serum Biomarker Chips. Eleven individual labeling reactions were performed on serum from a single breast cancer patient as well as serum from a healthy age and gender matched control. These labeled proteins were used to probe the S&S Serum Biomarker Chip, and the relative protein expression ratio for each of the 120 specificities was determined. The binding reaction was detected using both streptavidin-Dy647 and anti-fluorescien-Dy547 conjugates with the aid of an Axon Professional 4200A Scanner. Spot finding, background corrections, and two-channel data sampling was accomplished with ArrayVision™FAST® software. The SBC analysis workbook is a Microsoft® *Excel* file that converts fluorescent data into numerical values that represent the abundance of antigen in Sample A relative to antigen in Sample B. Average signal intensity was determined by taking the average of the sum of the Dy547 signal and the Dy647 signal for each triplicate on the array.

Two Color Reproducibility: The %CV of the protein expression ratios for 105 of the 120 specificities on the S&S Serum Biomarker chip is less than 20%. For those antibodies where the %CV is higher than 20% the total signal intensity from both Dy547 and Dy647 is often very low suggesting that these proteins are not present in detectable quantities in the serum samples tested.

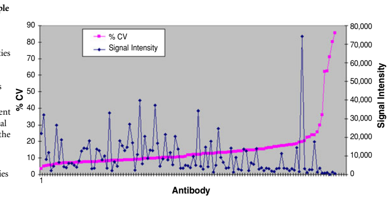
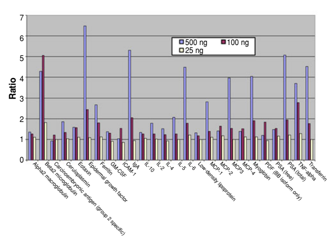


Figure 5. Limits of detection and Sensitivity of the Capture Antibodies on the Serum Biomarker Chip. A cocktail of 26 different purified human antigens were spiked into fetal calf serum (FCS) at three different concentrations; 500, 100 and 25 ng/ml final concentration. Four microliters of this spiked FCS was compared to un-spiked FCS using the two-color system of streptavidin DY647 and anti-fluorescien DY547 as described above. Spiked and un-spiked samples were labeled with biotin-ULS and fluorescien-ULS for 8 hours; spiked serum labeled with Biotin-ULS and un-spiked serum labeled with fluorescien-ULS were mixed and incubated with the Serum Biomarker Chip for 16 hours in pad A, while the dye swap combination was incubated in pad B (spiked serum labeled with fluorescien-ULS and un-spiked serum labeled with biotin-ULS). Data is expressed as the ratio of the average signal intensity of the spiked FCS to the un-spiked FCS. Average signal intensity was determined as described in Figure 4.

The S&S Serum Biomarker Chip detects purified human antigens spiked into a complex protein mixture (FCS). The greater the amount of protein spiked into the FCS the higher the observed ratio of that protein relative to the un-spiked FCS. As the concentration of spiked protein was decreased, the observed ratios decreased as well.



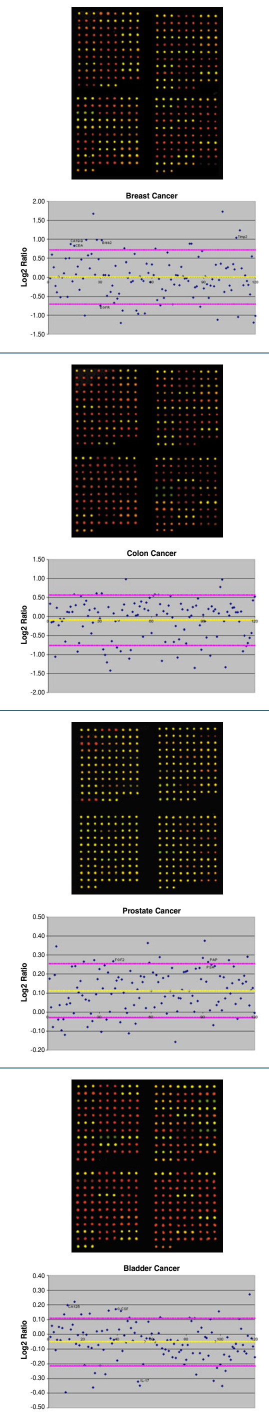


Figure 6: Scatter graphs of the protein expression ratios from breast, colon, bladder, and prostate cancer. Serum from a single breast, colon, bladder and prostate cancer patient, and serum from healthy age and gender matched controls were labeled with Biotin-ULS and Fluorescein-ULS, then pooled and probed against the Serum Biomarker Chip. Bound protein was detected with Dy647 streptavidin and Dy547 anti-fluorescien antibody followed by scanning on an Axon GenePix Professional 4200A Scanner. Spot finding, background corrections, and two-channel data sampling was accomplished with ArrayVision™FAST® software. The SBC analysis workbook is a Microsoft® Excel file that converts fluorescent data into numerical values that represent the abundance of antigen in Sample A relative to antigen in Sample B. A log2 transformation of the relative protein expression ratio for each of the 120 specificities is shown. The mean of all 120 ratios is shown as a yellow line and the purple lines represent 1.25 standard deviations away from the mean; 1.25 standard deviations was determined as the area where greater than 90% of all data points between self/self experiments lie. In an experimental situation where two different samples are compared, data points that fall outside the 1.25 SD boundry indicates a high likelihood for valid differences. Scatter graphs of the protein expression ratios from serum from 4 different cancer patients clearly show that the S&S Serum Biomarker Chip can detect differences in relative protein abundance between serum samples. A value of zero indicates that equal quantities of a particular protein are present in both samples. Values greater than zero indicate that the protein is more abundant in the cancer patient serum while a value of less than zero indicates that the protein is more abundant in the control serum. Those values that fall outside of 1.25 standard deviations from the mean are strong candidates for further investigation.

Summary

- The S&S Serum Biomarker Chip contains 120 antibodies to human serum biomarkers reproducibly printed in triplicate.
- Using the ULS-labeling system the %CV from multiple experiments was less the 20% for the majority of the elements on the S&S Serum Biomarker Chip
- The S&S Serum Biomarker Chip shows that human antigens bind specifically to the capture antibodies immobilized on the chip's surface.
- Purified human proteins spiked into non-human serum (FCS) were captured and detected by the Serum Biomarker Chip in a dose-dependent manner.
- The S&S Serum Biomarker Chip demonstrated differences in the relative protein abundances between serum samples from normal and cancer patients.

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