From aptamer-based biomarker discovery to diagnostic and clinical applications: an aptamer-based, streamlined multiplex proteomic assay

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Abstract

Recently, we reported an aptamer-based, highly multiplexed assay for the purpose of biomarker identification. To enable seamless transition from highly multiplexed biomarker discovery assays to a format suitable and convenient for diagnostic and life-science applications, we developed a streamlined, plate-based version of the assay. The plate-based version of the assay is robust, sensitive (sub-picomolar), rapid, can be highly multiplexed (up to 200 analytes), and fully automated. We demonstrate that quantification by microarray-based hybridization, Luminex bead-based methods, and qPCR are each compatible with our platform, further expanding the breadth of proteomic applications for a wide user community.

Introduction

Initial proteomic analyses using 2D gels were carried out in the 1970's [1]. Due to significant technical challenges [2], the promise of proteomics is only now being realized in biomarker discovery, life science research, pharmaceutical research and development, and medical diagnostics. We developed an aptamer-based multiplexed proteomic technology for biomarker discovery (the SOMAscan platform) and have successfully applied this technology to discover biomarker signatures in clinical studies of human diseases [3,4]. For high-content biomarker discovery, this technology was implemented with a bead-based assay on an automated robotic platform [5]. This discovery assay is highly multiplexed (>800 analytes), sensitive (sub-picomolar), reproducible, and quantitative [3]. Here we present a streamlined microtiter plate-based version of our proteomics discovery assay to provide a rapid, efficient and seamless transition from SOMAScan-developed biomarker panels identification to [3,4] to actual *in vitro* diagnostics small plex panels and specialized proteomics measurements that facilitate clinical drug development. We term this streamlined aptamer-based assay format a SOMAPanel.

Results

Principles of SOMAScan and SOMAPanel assays: At the core of the assay is an analyte capture reagent that consists of a fully synthetic aptamer coupled to a

biotin moiety through a photocleavable linker (Figure 1A). The biotin moiety permits binding to the streptavidin supports used for immobilization and wash steps, while the photocleavable linker permits release of the aptamer into solution after washing. A Cy3 fluorophore built into the capture reagents used in this study permits quantification by means of commercially available slide-based microarray hybridization systems, but is not required for all formats of the assay.

We note that while any aptamer with sufficient affinity and slow dissociation rate could be used in the proceeding manner, the aptamer reagents used in our published work are single-stranded DNA and bear dU residues each uniformly functionalized at the 5-position (e.g. benzyl, 2-napthyl, or 3-indolyl-carboxamide). These modified nucleotides greatly improve nuclease resistance, affinity, and selection success rate [6]. Such specialized aptamers are termed "SOMAmers" (Slow Off-rate Modified Aptamer). Here we use the generic term "aptamers", however, the data presented here was generated with SOMAmers exclusively. It should also be noted that in this work "PB-aptamer" refers to the photocleavable biotin aptamer capture reagent in its entirety, while "aptamer" refers to the aptamer component alone.

The assays themselves consist of a binding step in which PB-aptamers and analytes are equilibrated in solution (Figure 1B Panel 1), followed by immobilization of all PB-aptamers on a streptavidin-substituted support (Figure 1B Panel 2, "Catch-1"). Subsequent washes remove proteins that are not stably complexed with PB-aptamers. Proteins immobilized through interaction with bound PB-aptamers are biotinylated with an amine-reactive biotinylation reagent

(N-hydroxysuccinimide –PEO$_4$-biotin). After further washes, the entire aptamer population, including analyte-aptamer complexes, is released into solution via long-wave ultraviolet light-catalyzed cleavage of the biotin-bearing photocleavable linker (Figure 1B Panel 3). The biotinylated analyte-aptamer complexes are then selectively captured on another streptavidin support (Figure 1B Panel 4, "Catch-2") and the remaining, non-complexed aptamers are washed away. Finally, analyte-bound aptamers are eluted by disrupting the affinity interaction (Figure 1B Panel 5). Eluted aptamers are surrogates for analyte concentrations that can be quantified by standard DNA-quantification methods, for example, qPCR, or hybridization to microarrays (Figure 1B Panel 6).

To develop the plate-based SOMAPanel assay, a model multiplex consisting of nine PB-aptamers specific for the proteins IL-8, tPA, resistin, MIP-4, MMP-7, MMP-9, RANTES, MCP-1, and Lipocalin 2, and twenty control PB-aptamers, was assembled. These target analytes represent three broad ranges of abundance in plasma or serum, and their SOMAmer capture reagents were demonstrated as specific for their respective analytes by pull-down assay (Supplemental Figure 1). The twenty control PB-aptamers have no cognate analyte in serum or plasma and were used to monitor non-specific aptamer signaling.

Features of the plate-based SOMAPanel assay: Substitution of streptavidin plates for streptavidin-agarose beads and magnetic streptavidin beads eliminates both vacuum filtration and magnetic separation from the assay protocol. In

manual form, the assay becomes a "wash and dump" procedure that is reminiscent of an ELISA-based assay. The processing time is roughly 70 minutes. A diagram of the assay steps is shown in Figure 2A.

Dose-response curves with purified analytes generated in the plate-based assay performed manually are shown in Figure 2B. This 9-plex measurement compared assay response to increasing spiked-in analyte concentration as a function of PB-aptamer concentration in the presence of plasma. In general, little sensitivity is gained by elevated aptamer concentrations. We have chosen an intermediate concentration, 0.5 nM in each PB-aptamer, for the work shown here, though it is apparent from the curves that more or less PB-aptamer can be used in this particular analyte panel without significant penalty.

Semi-automation of the plate-based assay: It was anticipated that the "wash and dump" nature of the plate-based assay would permit automation using commercially available, relatively low-cost instrumentation intended for ELISA. The nature of the assay suggested that the additional capability of multiple reagent addition would permit near-complete automation. The commercially available BioTek EL406 was selected for this capacity. In addition to its conventional plate-washing capability, the EL406 supports addition of up to six different reagents, which is the number of solutions used in this assay.

Adaptation of the manual protocol for use with the EL406 was straightforward, and measurement of lower limits of quantification (LLOQ) in a 9-plex, semi-automated, plate-based assay revealed little loss in sensitivity or

stability relative to the bead-based SOMAScan assay (Figure 3 and supplemental materials, compare left and right panels).

The semi-automated, plate-based assay protocol proved considerably more rapid and convenient than the semi-automated bead-based assay. The post-equilibration processing time was reduced from one hundred fifty minutes to fifty minutes, thus allowing for greater (circa 4-fold) throughput. Hands-on operations for the semi-automated assay are limited to movement of plates from plate washer to UV lamp and back; and transfer of samples from one plate to another after photocleavage. Little attendance is required other than for these steps. Preparation and pipetting of beads is eliminated. A summary of these differences is presented in Table 1.

Nucleic acid quantification schemes: The bead-based assay and plate-based experiments shown up to this point use a nucleic acid quantification system based on hybridization to printed microarrays from Agilent to quantify aptamers in the final assay eluate. This system has the capacity to quantitatively measure more than 3000 analytes per sample, and even higher levels of multiplexing are certainly possible. It has proven sensitive and convenient for very highly multiplexed biomarker discovery applications. However, many labs have invested in other potentially suitable hybridization-based nucleic acid quantification instrumentation. Hence, we compared an alternative bead-based nucleic acid quantification platform with Agilent microarrays with respect to compatibility with our aptamer-based multiplex assay.

We measured limits of quantification of 9 analytes in multiplex format, in exactly the same manner as in Figure 3. We split the final eluates into two parts and independently determined limits of quantification using Agilent microarrays and the Luminex bead-based system as a final readout (Table 2). We found that sensitivity and dynamic ranges are roughly comparable between the two platforms, although Luminex was slightly less sensitive than Agilent (Table 2, compare columns 2 and 3), and exhibited slightly elevated upper limits of quantification (Table 2, compare columns 4 and 5).

Our biomarker discovery efforts have revealed that analyte concentration differences that distinguish case from control populations are often subtle. Indeed, we have discovered useful biomarkers that differ by as little as twenty percent between case and control [4]. To determine whether the plate-based assay in combination with a Luminex bead-based nucleic acid readout is suitable for measurements involving such subtle analyte concentration differences in the region of endogenous levels, we spiked in analytes in 20% increments, in quintuplicate, in the regions of analyte signal (and presumably, endogenous analyte concentrations) previously measured in serum titrations performed in the plate-based format (data not shown). It should be noted that listed analyte concentrations are nominal, based on protein mass as noted by the manufacturer without reference to purity, and hence cannot be used as standards to infer actual endogenous concentrations.

We find that even at these low levels of signal, subtle changes in concentration can be measured with good precision (Figure 4). The average CV for all analytes was 6.1%, with linear responses in the ranges tested.

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Validating the plate-based front end with alternative back-end readouts:
We wished to determine whether the plate-based assay, in combination with an alternative nucleic acid (aptamer) readout, is sufficiently sensitive and robust to separate case and control populations within a clinical sample set, which is one commercial diagnostic application anticipated for this assay. To this end we performed an experiment in which various analytes were spiked at levels comparable to those we have encountered in the course of biomarker discovery into a collection of individual serum samples, effectively creating a mock disease signature in a population of samples. The ability to distinguish differential expression of analytes, both up and down with respect to the control population, forms a practical criterion for the adequacy of the assay to discern target responses against the backdrop of individual sample variance.

Serum samples from twenty-four healthy controls were used to create a protein signature with both "up-" and "down-regulated" analytes. Two aliquots of each sample were used to produce a separate control and a case population by adding analytes to each group. Spikes into the twenty-four samples comprising the control group will result in "down-regulated" measurements in the case population while spikes into the case population will result in "up-regulation". We spiked three analytes into the control samples (tPA, MMP-9, and Lipocalin 2),

and four analytes in the case samples (IL-8, MCP-1, resistin and RANTES). The model multiplex assay was used to explore differential expression in this set of mock case and control samples.

The results are presented in Figure 5. Cumulative distribution functions (CDFs) were constructed separately for the case and control populations for each of the nine analytes. The three analytes spiked into the control group result in clearly identified "down-regulation", while the four analytes spiked into the case group appear as "up-regulation" in our mock protein signature. The two analytes, for which no spikes were added, MIP-4, and MMP-7, display no differential expression, attesting to the specificity of the aptamer assay. The magnitudes of the spiked proteins were relatively small to result in mostly overlapping distributions between case and control populations yet with discernable differences that are comparable to those observed in actual case/control proteomic studies [4]. The plate-based aptamer assay performed well in this model multiplex diagnostic application.

Discussion

We developed a streamlined multiplexed aptamer-based assay that is robust, sensitive, and quantitative. It is designed to enable the translation of discovery biomarker panels into robust diagnostic products and to facilitate use of small panels during clinical development of drugs. The ease of use of the assay is roughly comparable to that of single-analyte ELISA. The assay is easily

and inexpensively automated. Throughput can be made relatively high and sample volumes are quite small (~15 µL). Equipment and materials for the assay are commercially available from several sources. The final readout can be made inexpensive and scaled according to analyte number by use of a commercially available, bead-based nucleic acid quantification system.

Recently, we have identified several biomarker panels with potential diagnostic applications for chronic kidney disease [3], lung cancer [4], mesothelioma, and pancreatic cancer [in preparation]. Use of these biomarker panels in diagnostic applications will require the measurement of perhaps 9-15 analytes in a single sample. The streamlined assay presented here permits seamless transition from such biomarker panels identified in SOMAScan-based studies to actual diagnostic applications. We have demonstrated such utility by spiking analytes into a sample population to produce typical differential expression observed in proteomic studies of case/control groups.

We have found that the bead-based nucleic acid quantification system from Luminex can be used for final readout without significant performance penalty. This is advantageous in that it permits scaling of the final readout to the number of analytes to be measured. As such, the assay can be made more economical for applications involving specific small analyte panels. Moreover, the demonstration that several readout platforms may be used will render the aptamer-based assay easily accessible to groups and institutions that already possess other gene expression measurement platforms.

We have also explored the use of real-time quantitative PCR (qPCR) as a back-end read-out for the plate- and bead-based assays. As might be expected, qPCR is exquisitely sensitive, and can be made reproducible and quantitative with appropriate optimization (supplemental Figure 3 and data not shown). We conclude that qPCR is a viable back-end readout option for the aptamer-based assay presented here, and is certainly suitable for experimentation as well as routine assays in labs that possess the necessary equipment.

We have only briefly explored the upper limits of multiplex capacity of the plate-based assay but have verified that at least 60 analytes, selected from a lung cancer panel identified as biomarkers in an 836-plex SOMAScan assay, may be multiplexed without optimization (Supplemental Figure 4)

The use of aptamers as capture reagents carries advantages over traditional immunoassays developed with antibody sandwiches. The synthetic nature of aptamers ensures uniformity and availability. Customization of the affinity reagent is routine, relying only on the availability of the appropriate phosphoramidites. Aptamers are chemically stable and resistant to freeze-thaw cycles as well as heat denaturation. Custom generation of aptamers to protein targets is generally rapid and inexpensive compared to antibodies. The intrinsic limitations of multiplex capabilities of antibodies are greatly diminished with aptamers. To date, we have successfully multiplexed up to 836 aptamer measurements in a single 15 μL sample and do not anticipate an upper limit on multiplexing.

Materials and Methods

Purchased reagents: HEPES, NaCl, KCl, EDTA, EGTA, MgCl$_2$ and Tween-20 were purchased from Fisher Biosciences. Dextran sulfate sodium salt (DxSO$_4$), nominally 8000 molecular weight, was purchased from AIC and dialyzed against deionized water for at least 20 hours with one exchange. KOD EX DNA polymerase was purchased from VWR. Tetramethylammonium chloride and CAPSO were purchased from Sigma-Aldrich and streptavidin-phycoerythrin (SAPE) were purchased from Moss Inc. 4-(2-Aminoethyl)-benzenesulfonylfluoride hydrochloride (AEBSF) was purchased from Gold Biotechnology. Streptavidin-coated 96-well plates were purchased from Thermo Scientific (Pierce Streptavidin Coated Plates HBC, clear, 96-well, product number 15500 or 15501). NHS-PEO4-biotin was purchased from Thermo Scientific (EZ-Link NHS-PEO4-Biotin, product number 21329), dissolved in anhydrous DMSO, and stored frozen in single-use aliquots. IL-8, MIP-4, Lipocalin-2, RANTES, MMP-7, and MMP-9 were purchased from R&D Systems. Resistin and MCP-1 were purchased from PeproTech, and tPA was purchased from VWR.

Nucleic acids: Conventional (including amine- and biotin-substituted) oligodeoxynucleotides were purchased from Integrated DNA Technologies (IDT). Z-Block is a single-stranded oligodeoxynucleotide of sequence 5'- (AC-BnBn)$_7$-AC-3', where Bn indicates a benzyl-substituted deoxyuridine residue. Z-block was synthesized in-house, using conventional phosphoramidite chemistry. SOMAmer capture reagents were synthesized in-house by conventional

phosphoramidite chemistry, and purified on a 21.5 X 75 mm PRP-3 column, operating at 80℃ on a Waters Autopurification 2767 system (or Waters 600 series semi-automated system), using a timberline TL-600 or TL-150 heater and a gradient of triethylammonium bicarbonate (TEAB) / ACN to elute product. Detection was performed at 260 nm and fractions were collected across the main peak prior to pooling best fractions.

Buffers: Buffer SB18 is composed of 40 mM HEPES, 101 mM NaCl, 5 mM KCl, 5 mM $MgCl_2$, and 0.05% (v/v) Tween 20 adjusted to pH 7.5 with NaOH. Buffer SB17 is SB18 supplemented with 1 mM EDTA. Buffer PB1 is composed of 10 mM HEPES, 101 mM NaCl, 5 mM KCl, 5 mM $MgCl_2$, 1 mM trisodium EDTA and 0.05% Tween-20 adjusted to pH 7.5 with NaOH. CAPSO elution buffer consists of 100 mM CAPSO pH 10.0 and 1M NaCl. Neutralization buffer consists of 500 mM HEPES, 500 mM HCl, and 0.05% w/v Tween-20. Agilent Hybridization Buffer is a proprietary formulation that is supplied as part of a kit (Oligo aCGH/ChIP-on-chip Hybridization Kit). Agilent Wash Buffer 1 is a proprietary formulation (Oligo aCGH/ChIP-on-chip Wash Buffer 1, Agilent). Agilent Wash Buffer 2 is a proprietary formulation (Oligo aCGH/ChIP-on-chip Wash Buffer 2, Agilent). TMAC hybridization solution consists of 4.5 M tetramethylammonium chloride, 6 mM trisodium EDTA, 75 mM Tris-HCl (pH 8.0), and 0.15% (w/v) Sarkosyl. KOD buffer (10-fold concentrated) consisted of 1200 mM Tris-HCl, 15 mM $MgSO_4$, 100 mM KCl, 60 mM $(NH_4)_2SO_4$, 1% v/v Triton-X 100 and 1 mg/mL BSA

Sample preparation: Serum (stored at -80℃ in 100 µL aliquots), was thawed in a 25℃ water bath for 10 minutes, then stored on ice prior to sample dilution. Samples were mixed by gentle vortexing for 8 seconds. A 6% serum sample solution was prepared by dilution into 0.94× SB17 supplemented with 0.6 mM MgCl$_2$, 1 mM trisodium EGTA, 0.8 mM AEBSF, and 2 µM Z-Block. A portion of the 6% serum stock solution was diluted 10-fold in SB17 to create a 0.6% serum stock. 6% and 0.6% stocks are used to detect high- and low-abundance analytes, respectively.

Capture reagent (SOMAmer) and streptavidin plate preparation: SOMAmers were grouped into 2 mixes according to the relative abundance of their cognate analytes. Stock concentrations were 4 nM in each SOMAmer, and the final concentration of each SOMAmer was 0.5 nM. SOMAmer stock mixes were diluted 4-fold in SB17 buffer, heated to 95 ℃ for 5 min and cooled to 37℃ over a 15 minute period prior to use. This denaturation-renaturation cycle is intended to normalize SOMAmer conformer distributions and thus ensure reproducible SOMAmer activity in spite of variable histories. Streptavidin plates were washed twice with 150 µL buffer PB1 prior to use.

Equilibration and plate capture: Heat-cooled 2× SOMAmer mixes (55 µL) were combined with an equal volume of 6% or 0.6% serum dilutions, producing equilibration mixes containing 3% and 0.3% serum. The plates were sealed with a Silicone Sealing Mat (Axymat Silicone sealing mat, VWR) and incubated for 1.5 h at 37 ℃. Equilibration mixes were then transferred to the wells of a washed 96-well streptavidin plate and further incubated on an Eppendorf Thermomixer set at 37 ℃, with shaking at 800 rpm, for two hours.

Manual Assay: Unless otherwise specified, liquid was removed by dumping, followed by two taps onto layered paper towels. Wash volumes were 150 μL and all shaking incubations were done on an Eppendorf Thermomixer set at 25 °C, 800 rpm. Equilibration mixes were removed by pipetting, and plates washed twice for 1 minute with buffer PB1 supplemented with 1 mM dextran sulfate and 500 μM biotin, then 4 times for 15 seconds with buffer PB1. A freshly made solution of 1 mM NHS-PEO$_4$-biotin in buffer PB1 (150 μL /well) was added, and plates incubated for 5 minutes with shaking. The NHS-biotin solution was removed, and plates washed 3 times with buffer PB1 supplemented with 20 mM glycine, and 3 times with buffer PB1. Eighty-five μL of buffer PB1 supplemented with 1 mM DxSO$_4$ were then added to each well, and plates were irradiated under a BlackRay UV lamp (nominal wavelength 365 nm) at a distance of 5 cm for 20 minutes with shaking. Samples were transferred to a fresh, washed streptavidin-coated plate, or an unused well of the existing washed streptavidin plate, combining high and low sample dilution mixtures into a single well. Samples were incubated at room temperature with shaking for 10 minutes. Unadsorbed material was removed and the plates washed 8 times for 15 seconds each with buffer PB1 supplemented with 30% glycerol. Plates were then washed once with buffer PB1. SOMAmers were eluted for 5 minutes at room temperature with 100 μL CAPSO elution buffer. 90 μL of the eluate was transferred to a 96-well HybAid plate and 10 μL neutralization buffer was added.

Semi-Automated Assay: Streptavidin plates bearing adsorbed equilibration mixes were placed on the deck of a BioTek EL406 plate washer, which had been programmed to perform the following steps: unadsorbed material is removed by aspiration, and wells

are washed 4 times with 300 μL of buffer PB1 supplemented with 1 mM dextran sulfate and 500 μM biotin. Wells are then washed 3 times with 300 μL buffer PB1. One hundred fifty μL of a freshly prepared (from a 100 mM stock in DMSO) solution of 1 mM NHS-PEO4-biotin in buffer PB1 is added. Plates are incubated for 5 minutes with shaking. Liquid is aspirated, and wells were washed 8 times with 300 μL buffer PB1 supplemented with 10 mM glycine. One hundred μL of buffer PB1 supplemented with 1 mM dextran sulfate are added. After these automated steps, plates were removed from the plate washer and placed on a thermoshaker mounted under a UV light source (BlackRay, nominal wavelength 365 nm) at a distance of 5 cm for 20 minutes. The thermoshaker was set at 800 rpm and 25 °C. After 20 minutes irradiation, samples were manually transferred to a fresh, washed streptavidin plate (or to an unused well of the existing washed plate). High-abundance (3% serum + 3% aptamer mix) and low-abundance reaction mixes (0.3% serum + 0.3% aptamer mix) were combined into a single well at this point. This "Catch-2" plate was placed on the deck of BioTek EL406 plate washer, which had been programmed to perform the following steps: the plate was incubated for 10 minutes with shaking. Liquid is aspirated, and wells are washed 21 times with 300 μL buffer PB1 supplemented with 30% glycerol. Wells are washed 5 times with 300 μL buffer PB1, and the final wash is aspirated. One hundred μL CAPSO elution buffer are added, and SOMAmers are eluted for 5 minutes with shaking. Following these automated steps, the plate was then removed from the deck of the plate washer, and 90 μL aliquots of the samples were transferred manually to the wells of a HybAid 96-well plate that contained 10 μL neutralization buffer.

Hybridization to custom Agilent 8× 15k microarrays: 24 µL of the neutralized eluate were transferred to a new 96-well plate and 6 µL of 10× Agilent Block (Oligo aCGH/ChIP-on-chip Hybridization Kit, Large Volume, Agilent 5188-5380), containing a set of hybridization controls composed of 10 Cy3 SOMAmers was added to each well. Thirty µL 2× Agilent Hybridization buffer were added to each sample and mixed. Forty µL of the resulting hybridization solution were manually pipetted into each "well" of the hybridization gasket slide (Hybridization Gasket Slide, 8-microarray per slide format, Agilent). Custom Agilent microarray slides, bearing 10 probes per array complementary to 40 nucleotide random region of each SOMAmer with a 20× dT linker, were placed onto the gasket slides according to the manufacturers' protocol. The assembly (Hybridization Chamber Kit – SureHyb-enabled, Agilent) was clamped and incubated for 19 hours at 60 ℃ while rotating at 20 rpm.

Post Hybridization Washing: Approximately 400 mL Agilent Wash Buffer 1 was placed into each of two separate glass staining dishes. Slides (no more than two at a time) were disassembled and separated while submerged in Wash Buffer 1, then transferred to a slide rack in a second staining dish also containing Wash Buffer 1. Slides were incubated for an additional 5 minutes in Wash Buffer 1 with stirring. Slides were transferred to Wash Buffer 2 pre-equilibrated to 37℃ and incubated for 5 minutes with stirring. Slides were transferred to a fourth staining dish containing acetonitrile, and incubated for 5 minutes with stirring.

Microarray Imaging: Microarray slides were imaged with an Agilent G2565CA Microarray Scanner System, using the Cy3-channel at 5 µm resolution at 100% PMT setting, and the XRD option enabled at 0.05. The resulting TIFF images were

processed using Agilent feature extraction software version 10.5.1.1 with the GE1_105_Dec08 protocol.

Luminex probe design: Probes immobilized to beads bore 40 deoxynucleotides complementary to the 3' end of the 40 nucleotide random region of the target SOMAmer. The SOMAmer complementary region was coupled to Luminex Microspheres through a hexaethyleneglycol (HEG) linker bearing a 5' amino terminus. Biotinylated detection deoxyoligonucleotides consisted of 17-21 deoxynucleotides complementary to the 5' primer region of target SOMAmers. Biotin moieties were appended to the 3' ends of detection oligos.

Coupling of probes to Luminex Microspheres: Probes were coupled to Luminex Microplex Microspheres essentially per the manufacturer's instructions, but with the following modifications: amino-terminal oligonucleotide amounts were 0.08 nMol per 2.5×10^6 microspheres, and the second EDC addition was 5 µL at 10 mg/mL. Coupling reactions were performed in an Eppendorf ThermoShaker set at 25°C and 600 rpm.

Microsphere hybridization: Microsphere stock solutions (about 40000 microspheres/µL) were vortexed and sonicated in a Health Sonics ultrasonic cleaner (Model: T1.9C) for 60 seconds to suspend the microspheres. Suspended microspheres were diluted to 2000 microspheres per reaction in 1.5✕ TMAC hybridization solutions and mixed by vortexing and sonication. Thirty-three µL per reaction of the bead mixture were transferred into a 96-well HybAid plate. Seven

μL of 15 nM biotinylated detection oligonucleotide stock in 1× TE buffer were added to each reaction and mixed. Ten μL of neutralized assay sample were added and the plate was sealed with a silicon cap mat seal. The plate was first incubated at 96 °C for 5 minutes and incubated at 5 0 °C without agitation overnight in a conventional hybridization oven. A filter plate (Dura pore, Millipore part number MSBVN1250, 1.2 μm pore size) was prewetted with 75 μL 1× TMAC hybridization solution supplemented with 0.5% (w/v) BSA. The entire sample volume from the hybridization reaction was transferred to the filter plate. The hybridization plate was rinsed with 75 μL 1× TMAC hybridization solution containing 0.5% BSA and any remaining material was transferred to the filter plate. Samples were filtered under slow vacuum, with 150 μL buffer requiring about 8 seconds to evacuate. The filter plate was washed once with 75 μL 1× TMAC hybridization solution containing 0.5% BSA and the microspheres in the filter plate were resuspended in 75 μL 1× TMAC hybridization solution containing 0.5% BSA. The filter plate was protected from light and incubated on an Eppendorf Thermalmixer R for 5 minutes at 1000 rpm. The filter plate was then washed once with 75 μL 1× TMAC hybridization solution containing 0.5% BSA. 75 μL of 10 μg/mL streptavidin phycoerythrin (SAPE-100, MOSS, Inc.) in 1× TMAC hybridization solution was added to each reaction and incubated on Eppendorf Thermalmixer R at 25°C at 1000 rpm for 60 minutes. The filter plate was washed twice with 75 μL 1× TMAC hybridization solution containing 0.5% BSA and the microspheres in the filter plate were resuspended in 75 μL 1× TMAC hybridization solution containing 0.5% BSA. The filter plate was then

incubated protected from light on an Eppendorf Thermalmixer R for 5 minutes, 1000 rpm. The filter plate was then washed once with 75 μL 1× TMAC hybridization solution containing 0.5% BSA. Microspheres were resuspended in 75 μL 1× TMAC hybridization solution supplemented with 0.5% BSA, and analyzed on a Luminex 100 instrument running XPonent 3.0 software. At least 100 microspheres were counted per bead type, under high PMT calibration and a doublet discriminator setting of 7500 to 18000.

QPCR read-out: Standard curves for qPCR were prepared in water ranging from 10^8 to 10^2 copies with 10-fold dilutions and a no-template control. Neutralized assay samples were diluted 40-fold into diH_2O. The qPCR master mix was prepared at 2× final concentration (2× KOD buffer, 400 μM dNTP mix, 400 nM forward and reverse primer mix, 2× SYBR Green I and 0.5 U KOD EX). Ten μL of 2× qPCR master mix was added to 10 μL of diluted assay sample. qPCR was run on a BioRad MyIQ iCycler with 2 minutes at 96 °C followed by 40 cycles of 96 °C for 5 seconds and 72 °C for 30 seconds.

Pull-down assay: Pull-down assays were performed as described previously[3].

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Figure Legends

Figure 1. Aptamer-based assay reagent and assay principles. The

primary analyte capture reagent and quantified component consists of an

analyte-specific aptamer coupled to a Cy3 moiety and a biotin group joined to

the fluorophore-substituted aptamer through a photocleavable linker (Panel

A). The principal features of the assay consist of equilibration of capture

reagent and analyte mixture in solution, followed by immobilization of the

entire capture reagent population on immobilized streptavidin through the

biotin moieties of the capture reagent population. The immobilized capture

reagent population, including analyte/capture reagent complexes, is washed

to remove non-complexed proteins. Immobilized protein analytes are then

biotinylated using a conventional amine-reactive biotinylation reagent. The

entire capture reagent population, including biotinylated analyte/capture

reagent complexes, is then released into solution via photocleavage.

Biotinylated analyte/capture reagent complexes are exclusively captured on

immobilized streptavidin via the biotin moieties appended to the analyte

population. Washes remove the capture reagent population at large, leaving

only analyte/capture reagent complexes. The remaining capture reagent

population is a surrogate for the analyte/capture reagent population. This

material is eluted from immobilized analytes and quantified via conventional

DNA quantification methods.

Figure 2. Diagram of manual plate-based assay steps and dose-response curves generated in manual plate-based assay format. The five

steps of the manual plate-based assay - equilibration, biotinylation,

photocleavage, and elution, are punctuated by three wash-and-dump cycles

and one liquid transfer. The total processing time is about 70 minutes (Panel

A). A set of dose response curve generated in a nine-plex manual assay

format at various capture reagent concentrations (Panel B). Dose-response

curves were generated by spiking analytes into plasma at the indicated

concentrations. Shown are resistin and MCP-1. Dose-response curves of

MIP-4, RANTES, MMP-9, MMP-7, Lipocalin 2, tPA, and IL-8 can be found in Supplemental Materials.

Figure 3. Precision profiles and limits of quantification of plate- and bead-based assays. Eight individual measurements of fluorescent signal as a function of analyte concentration in buffer were made for each of nine analytes in multiplexed format. For the dose-response curves (left of each panel), the average RFU at each concentration is denoted by the blue markers and the eight individual measurements used to compute each average are denoted by the red markers plotted on the four parameter curve fit (solid blue line). Precision profiles (right of each panel) were computed with two different methods: (1) by calculating the variance in computed concentrations (blue, bottom left of each panel) and (2) by calculating the variance in log RFU (assay response, top right of each panel) combined with the slope of the standard curve (red). Panels A, C, and E were generated in plate-based format. Panels B, D, and F were generated in bead-based format. Analytes measured were MCP-1 (Panels A and B), MMP9 (Panel C and D), resistin (Panels E and F) tPA (Supplemental Figure 1B), MMP-7 (Supplemental Figure 1B), IL-8 (Supplemental Figure 1A), Lipocalin 2 (Supplemental Figure 1A), MIP-4 (Supplemental Figure 1A), Protein S (Supplemental Figure 1B) and RANTES (Supplemental Figure 1B).

Figure 4. **High-resolution titration of analytes.** Analytes were titrated in 20% concentration increments in the region of signal generated by serum without spikes. Assay eluates were quantified by Luminex bead hybridization.

Figure 5. Differential expression between case and control populations. Twenty-four case samples and twenty-four control samples were measured using the model 9-plex plate-based assay. Empirical CDFs were constructed for the control (blue) and case (red) populations separately for each analyte and are displayed in panels a-i. Spikes into the control samples (tPA, MMP-9, and Lipocalin 2) result in clear "down-regulation", spikes into case samples (IL-8, MCP-1, resistin and RANTES) result in clear "up-regulation" and the two analytes not spiked (MIP-4 and MMP-7) show no differential expression.

<div align="center">

Supporting Figure Legends

</div>

Supporting Figure 1. **Demonstration of aptamer specificity by pull-down assay**. SOMAmers were incubated with target proteins, plasma, or target proteins spiked into plasma for 45 minutes. Protein/SOMAmer complexes were captured on magnetic streptavidin beads (MyOne C1), washed, and then treated with a mixture of NHS-biotin and NHS-AlexaFluor 647. Protein/SOMAmer complexes were photocleaved from beads and a portion fractionated on SDS gels (first set of 3 lanes, marked "equilibrium"). Protein/SOMAmer complexes

were then adsorbed to monomeric avidin agarose beads, washed, and then

eluted with 2 mM biotin in SB17. Complexes were captured a third time onto

magnetic streptavidin beads (MyOne C1) substituted with a bound biotinylated-

primer complementary to the 3' fixed region of the SOMAmer. Not all SOMAmer

complexes can be captured onto these beads since the 3' fixed regions of

SOMAmers are sometimes inaccessible for annealing while bound to the target

protein (as evident in the gels for MMP-7 and MMP-9). The complexes were

eluted by increasing the pH to 12, and then neutralized. Portions were

fractionated on SDS gels (second set of 3 lanes). Shown are purified target

protein spiked into buffer (lanes 1), purified target protein spiked into 10% plasma

(lanes 2), and 10% plasma with no spike (lanes 3).

**Supporting Figure 2. Precision profiles and limits of quantification of plate-
and bead-based assays.** Eight individual measurements of fluorescent signal

as a function of analyte concentration in buffer were made for each of nine

analytes in multiplexed format. For the dose-response curves (left of each panel),

the average RFU at each concentration is denoted by the blue markers and the

eight individual measurements used to compute each average are denoted by

the red markers plotted on the four parameter curve fit (solid blue line). Precision

profiles (right of each panel) were computed with two different methods: (1) by

calculating the variance in computed concentrations (blue, bottom left of each

panel) and (2) by calculating the variance in log RFU (assay response, top right

of each panel) combined with the slope of the standard curve (red). Left-hand

panels were generated in plate-based (SOMAPanel) format. Right-hand panels were generated in bead-based (SOMAscan) format.

Supporting Figure 3. qPCR readout of plate-based assay eluates. Portions of samples generated in the experiment for Table 2 were diluted and assayed by qPCR per Materials and Methods.

Supporting Figure 4. Minimum multiplex capacity of plate-based assay platforms. Sixty-one SOMAmers recognizing analytes identified as biomarkers on the bead-based SOMAScan platform were combined. Serum was added at 0.011%, 0.035%, 0.11%, 0.35%, 1.1%, 3.45%, 10.9%, and 34.5% v/v, and analyte signal measured as described in Materials and Methods. The log of the ratio of analyte signal at 10.9% and 0.011% was calculated, and plotted as a cumulative distribution function. Analyte signals at 10.9% serum are elevated at least 2.8-fold over those at 0.011% serum for all sixty-one biomarkers.

Tables

Table 1. Comparison of automated bead-based Discovery-plex and plate-based SOMAPanel assay formats.

Metric	Bead-based Discovery Assay	Plate-based SOMAPanel Targeted Assay
Partitioning Method (Capacity)	Catch 1 – SA Agarose Beads (>1000-plex)	Catch 1 & 2 – SA plates (~200-plex)
Up-front Prep (Time)	Bead prep (~30 minutes) Robotic setup	None
Post-equilibration processing time	~150 minutes	~50 minutes
Throughput	96 samples/day/FTE	384 samples/day/FTE
Manual operation	Yes	Yes
Average LLOQ	<1 pM	<2pM
Coefficient of variation	~5%	~7%
Automation instrumentation	Biomek FX with modifications	Stock BioTek EL406 plate washer

Table 2. Comparison of slide-based and bead-based read-out formats.

Analyte	Lower Limit of Quantification (pM)		Upper Limit of Quantification (pM)		Quantification Range (logs)	
	Agilent	Luminex	Agilent	Luminex	Agilent	Luminex
IL-8	0.32	0.5	210	240	2.8	2.7
MIP-4	0.66	2.0	3,600	1000,000	3.8	5.7
Lipocalin-2	0.83	0.78	260	1,500	2.5	3.3
MCP-1	1.2	1.8	700	1,500	2.8	3.9
RANTES	1.8	3.5	420	360	2.4	2.0
MMP-7	1.9	6.8	550	1,100	2.5	2.2
resistin	1.4	1.8	1,900	4,400	3.1	3.4
MMP-9	1.6	5.4	13,000	19,000	3.9	3.5
tPA	1.2	3.2	1,300	1,400	3.1	2.7

Figures

Figure 1A.
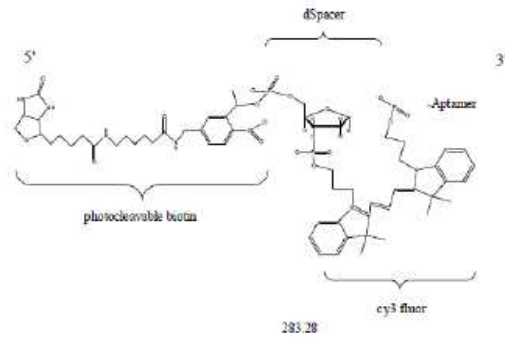
Figure 1B.

1. Bind

2. Catch 1

3. Cleave

4. Catch 2

5. Elute

6. Quantify
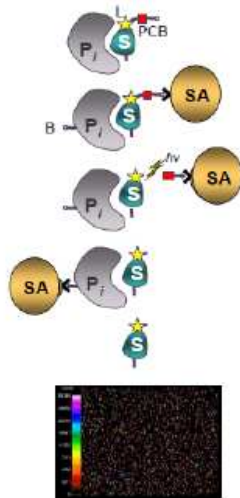
1. Equilibration in solution: SOMAmers (S) bind to cognate proteins (P_i)

2. Complexes immobilized on streptavidin ("Catch-1"). Free proteins washed away, and bound proteins tagged with biotin (B)

3. Kinetic challenge, release from streptavidin by photocleavage of UV-sensitive linker (PC)

4. Complexes immobilized on streptavidin ("Catch 2").

5. Additional washing, release of SOMAmers

6. Detect SOMAmers by standard hybridization-based DNA quantification.
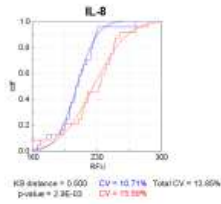
Figure 2A
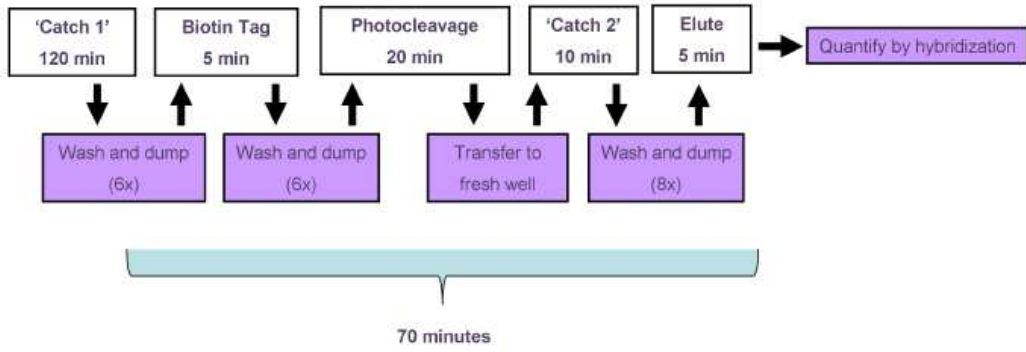
Figure 2B
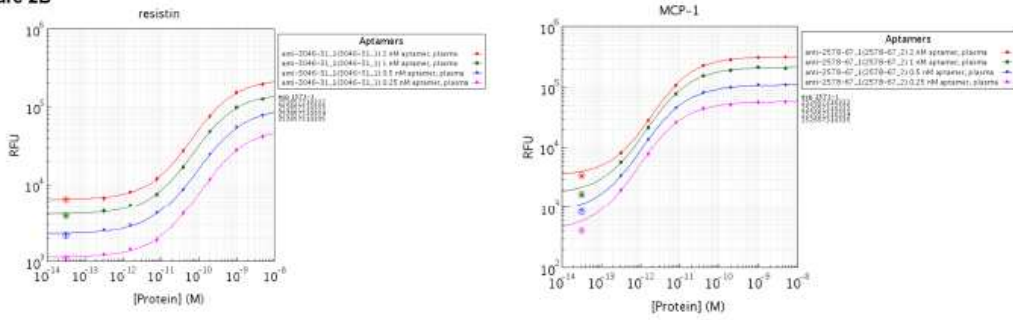
Figure 3.
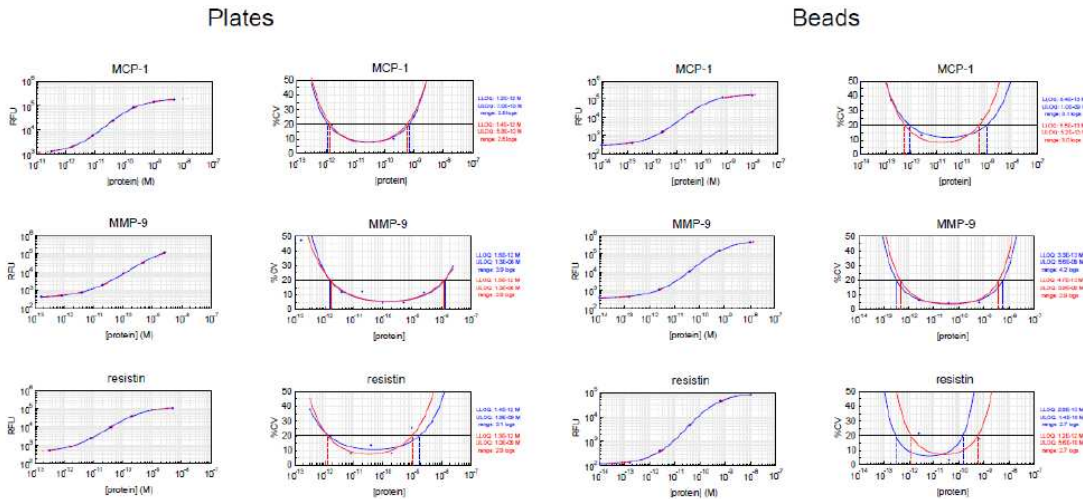
Figure 4.
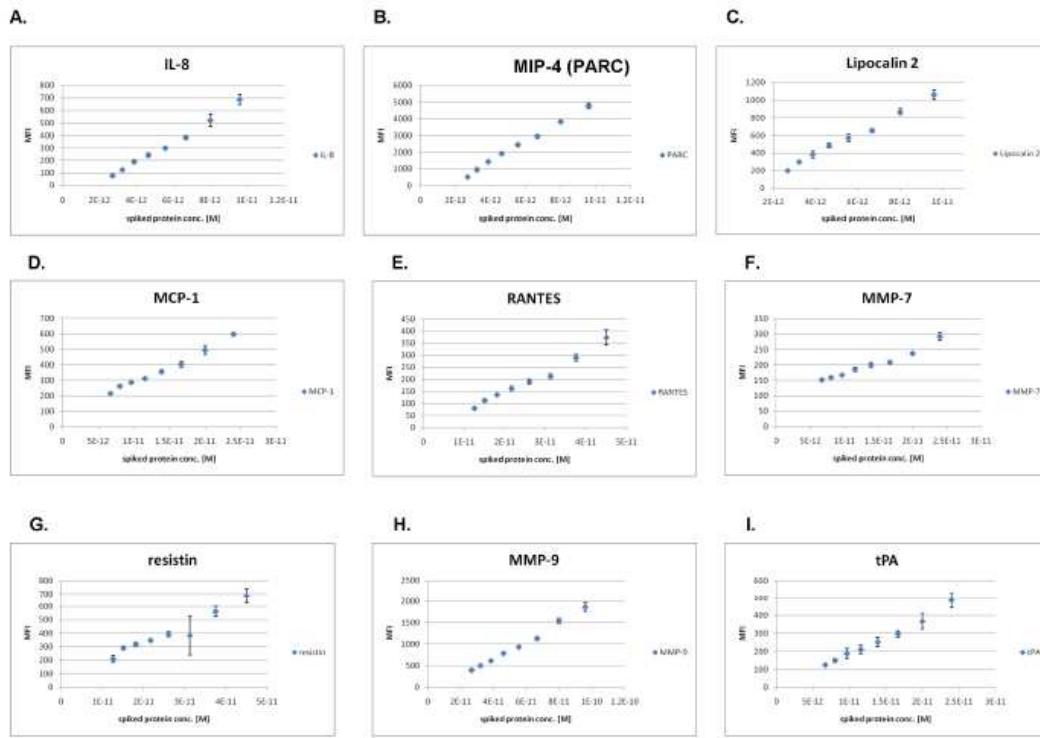
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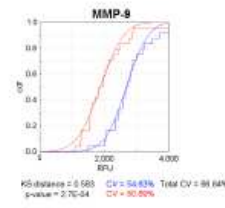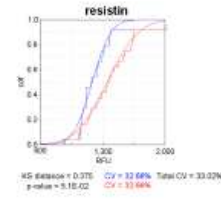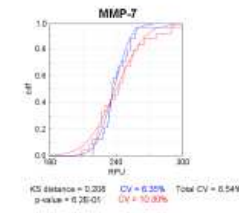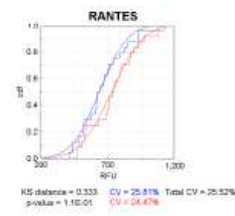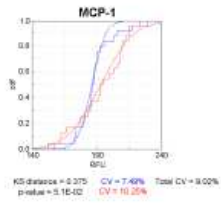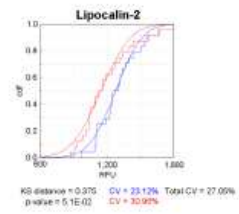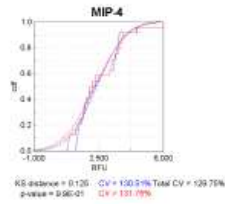
Supporting Figure 1.
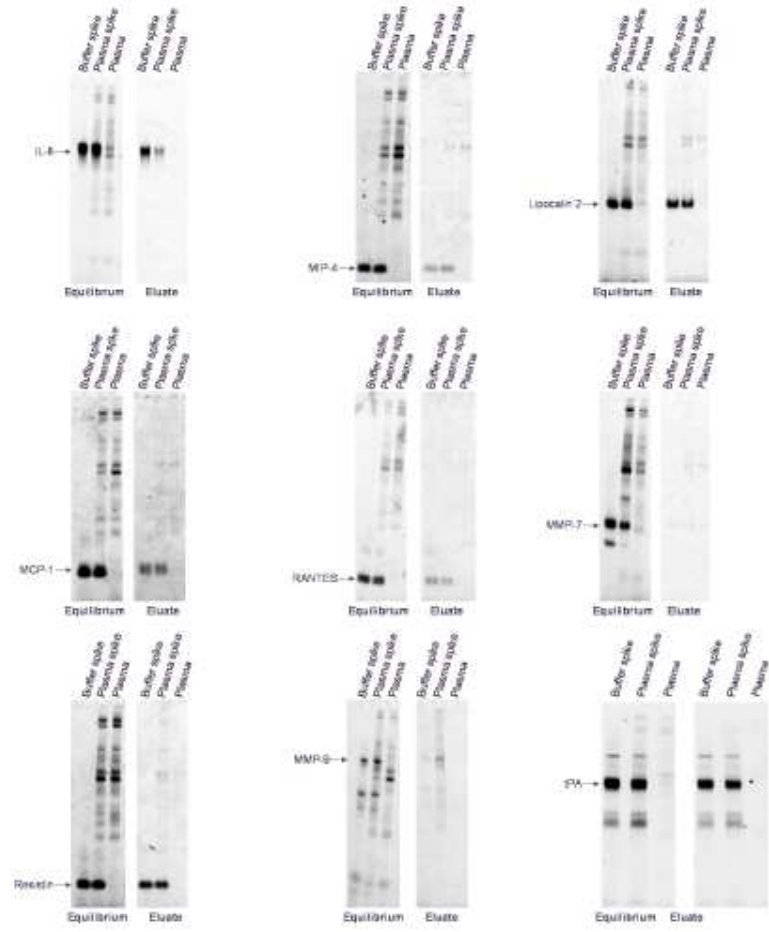
Supporting Figure 2.

Supporting Figure 3.

Cumulative distribution of serum response of 61-plex