Toward Next Generation Plasma Profiling via Heat-induced Epitope Retrieval and Array-based Assays*

Jochen M. Schwenk‡§, Ulrika Igel‡¶, Maja Neiman‡¶, Hanno Langen‖, Charlotte Becker**, Anders Bjartell‡‡, Fredrik Ponten§§, Fredrik Wiklund¶¶, Henrik Grönberg¶¶, Peter Nilsson‖, and Mathias Uhlen‡¶

There is a great need for high throughput methods for screening patient samples in the quest for potential biomarkers for diagnostics and patient care. Here, we used a combination of undirected target selection, antibody suspension bead arrays, and heat-induced epitope retrieval to allow for protein profiling of human plasma in a novel and systematic manner. Several antibodies were found to reveal altered protein profiles upon epitope retrieval at elevated temperatures with limits of detection improving into lower ng/ml ranges. In a study based on prostate cancer patients, several proteins with differential profiles were discovered and subsequently validated in an independent cohort. For one of the potential biomarkers, the human carnosine dipeptidase 1 protein (CNDP1), the differences were determined to be related to the glycosylation status of the targeted protein. The study shows a path of pursuit for large scale screening of biobank repositories in a flexible and proteome-wide fashion by utilizing heat-induced epitope retrieval and using an antibody suspension bead array format. Molecular & Cellular Proteomics 9: 2497–2507, 2010.

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There is a great need for protein biomarkers for early diagnosis of disease as well as for prognostic markers in which the outcome of a particular disease or treatment can be predicted (1). In particular, biomarkers that make it possible to monitor the progress of treatment or the reoccurrence of a particular disease are of great clinical value. However, there are still few protein biomarkers in clinical practice today, and despite many biomarker discovery efforts by many laboratories using many different approaches, a limited number have been introduced into the clinical routine during the last 10 years (2). The complexity of serum or plasma proteomes with their broad dynamic range of protein concentrations and the lack of high throughput methods with high sensitivity have hampered such discovery and validation efforts.

The most common approach for protein biomarker discovery today is the use of proteomics methods in which samples from case-control groups are compared using biochemical and biophysical methods, most notably with mass spectrometry (3). The introduction of more and more sophisticated instrumentation has increased the sensitivity and throughput of mass spectrometry during the last years (4). One of the advantages with mass spectrometry is that the method also allows for the detection of differences in protein modifications, such as glycosylation or phosphorylation, which have been found useful for some applications (5). Although many potential biomarkers have been discovered using mass spectrometry, the approach is yet limited to the analysis of a relatively small number of patient samples.

The alternative approach for biomarker discovery is to use affinity probes, usually antibodies but also other reagents, such as aptamers (6) or Affibody molecules (7). The advantage of such probe-based methods is the possibility to analyze many samples in parallel, and many assays based on antibodies, such as ELISA, are very sensitive in the sub-ng/ml range. In particular, sandwich immunoassays in which two separate antibodies are used to increase the sensitivity and selectivity allow proteins to be assayed down to pg/ml (8). Recently, new assays based on amplification methods have been described, such as the proximity ligation method (9), and these have the potential to score protein on a single molecule level. However, the lack of validated antibodies to most human proteins (10) makes it impossible to use antibody-based protocols for a majority of the potential protein targets, and this is even more difficult for assays based on paired antibodies that require two distinct antibodies with separate and non-overlapping epitopes. Because of this limitation, current studies are directed by candidate target lists reported in the...
Plasma Profiling by Heat-induced Epitope Retrieval

Recent efforts have been described for the generation of antibodies on a whole-proteome level (14). Version 6 of the Human Protein Atlas contains validated antibodies toward proteins from 8,400 human genes, corresponding to 42% of the protein-encoded genes in man. All antibodies published in the Human Protein Atlas are publicly available and include a total of more than 40 antibody providers from the United States, Canada, Europe, Australia, and Asia. Several other efforts, such as the ProteomeBender (15), the SH2 consortium (16), and the NCI affinity capture project (17), have recently been initiated with the aim to generate affinity reagents toward human protein targets. The objective of these efforts is to have publicly available antibodies to a representative protein from all of the protein-encoded genes by 2014 (18), and this emphasizes the need to develop high throughput methods for immunobased protein profiling to leverage this tool box of antibodies to allow high throughput biomarker discovery.

We have shown earlier that antibodies utilized in suspension bead arrays can be used for profiling proteins in serum and plasma (19). Hereby, we found that the ability to detect proteins such as components of the complement system was enhanced by heat treatment, most likely because epitopes might be exposed at elevated temperatures and thus become available for antibody binding. In particular, this is likely to be the case for antibodies recognizing linear epitopes on the protein target. Here, we analyzed the functionality of antibodies following different epitope retrieval protocols at different temperatures, and we describe a method for multiplex analysis of plasma or serum using plasma from patients with elevated PSA levels as an example. A method suitable for analysis of large numbers of biobank samples is presented.

MATERIALS AND METHODS

Antibodies—Protocols for antigen selection, cloning, expression, purification, and immunization of rabbits, followed by affinity purification to yield mono-specific polyclonal antibodies, and their characterization with Western blots and antigen microarrays were applied as described previously (20, 21). All protein fragments used for immunization were produced with a His6-albumin-binding protein tag and a 100-plex bead mixture as described previously (19). Bead coupling—Antibodies were coupled to carboxylated beads (Luminex Corp.) according to the manufacturer’s protocol and as described previously (19). The coupling efficiency for each antibody was determined via R-phycocerythrin-labeled anti-rabbit IgG antibody (Jackson ImmunoResearch Laboratories) and a 100-plex bead mixture was created as described previously (23).

Sample Labeling and Assay Procedure—Serum and plasma samples were labeled and analyzed in accordance with previous studies (19, 24) with minor changes. At first, samples were thawed at RT, centrifuged for 10 min at 10,000 rpm, and transferred into a microtiter plate (Abgene). The plates were centrifuged (1 min at 3,000 rpm), and 3 μl of each sample was added to 24.5 μl of 1× PBS with a liquid handler (Matrix PlateMate 2 × 2). N-Hydroxysuccinimidyl ester of biotinoyl tetraoxapentadecanoic acid (Pierce) was then added at 10-fold molar excess to yield an overall 1:10 sample dilution followed by a 2-h incubation at 4°C in a microtiter plate shaker (Thermomixer, Eppendorf). The reaction was stopped by the addition of a 250-fold molar excess of Tris-HCl, pH 8.0 over biotin and incubated for another 20 min at 4°C prior to a final storage at −80°C.

All samples were subsequently utilized without removing unincorporated biotin and diluted 1:50 (Matrix PlateMate) in an assay buffer composed of 0.5% (w/v) polyvinyl alcohol and 0.8% (w/v) polyvinylpyrrolidone (Sigma) in 0.1% casein in PBS supplemented with 0.5 mg/ml rabbit IgG (Bethyl Laboratories). The samples were treated in a thermocycler for 30 min at 23, 45, or 56°C for 15 min at 72°C; or 5 min at 96°C. Then, 45 μl was added to 5 μl of bead mixtures in a filter-bottomed microtiter plate (Millipore), and incubation took place overnight on a shaker at an ambient temperature. Beads were washed in wells with 3 × 50 μl of PBST (1× PBS, pH 7.4, 0.1% Tween20) on a vacuum device (Millipore) followed by 10 min with 50 μl of a stop solution containing 0.1% paraformaldehyde in PBS. Beads were washed with 1 × 50 μl of PBST, and 30 μl of 0.5 μg/ml R-phycocerythrin-labeled streptavidin (Invitrogen) in PBST was added and incubated for 20 min. Finally, wells were washed with 3 × 50 μl and measured in 150 μl of PBST.

Western Blot—Plasma samples were depleted of serum albumin and IgG by the use of Affibody molecules (Affibody AB) coupled to SulfoLink matrix (Pierce) as described elsewhere (25). Samples were diluted 1:10 in PBS, and the flow-through was applied per lane to SDS gel separation (4–12%) and BiTris (2-[bis[2-hydroxyethyl]amino]-2-[hydroxyethyl]propane-1,3-diol) as described previously (23).

Samples—Plasma spiked with recombinant PSA (R&D Systems) was prepared and provided by Roche, Basel, Switzerland. From Skåne University Hospital, Malmö, Sweden, 84 heparinized plasma samples donated from anonymized individuals were obtained. From these, total PSA (tPSA) values and free-to-total PSA ratios had been determined during clinical routine analysis. The samples were grouped based on these clinical parameters (supplemental Table 2) and used during discovery. For validation, a population-based case-control study of prostate cancer etiology, CAPS (Cancer Prostate in Sweden), was used. The study design has been described in detail elsewhere (22). All participants gave written informed consent, and the Research Ethics Committees at Karolinska Institute and Umeå University approved this investigation. Hereof, 90 EDTA plasma samples were used and defined as aggressive, fulfilling one of the following criteria: tumor stage T3/T4 or N1, Gleason sum 8 and above, or PSA >50 ng/ml. Less aggressive cases were classified as T1/T2 and N0/NX or M0/MX, PSA <50 ng/ml, and Gleason sum 4–7. Control samples were from prostate cancer-free males.

**Abbreliations used are:** PSA, prostate-specific antigen; LOD, limit of detection; LOQ, limit of quantification; tPSA, total PSA; HPA, Human Protein Atlas; HCRTR1, orexin receptor type 1; CNDP1, carnosine dipeptidase 1 protein; TMEM59L, transmembrane protein 59-like; BisTris, 2-[bis[2-hydroxyethyl]amino]-2-[hydroxyethyl]propane-1,3-diol.
performed using HRP-labeled anti-rabbit antibody (Dako) and a chemiluminescent substrate (Immobilon, Millipore).

Design, Readout, and Statistical Analysis—The sample location was randomized throughout a microtiter plate, and measurements were performed using an LX200 instrument (Luminex Corp.). At least 50 events per bead identity were counted, and binding events were displayed as median fluorescence intensity. Data analysis was performed using R (26), and the presented median fluorescence intensity values were processed by probabilistic quotation normalization as described earlier (27). Pearson correlation was used, and a limit of detection (LOD) was determined via a five-parametric logistic regression function to reveal the antigen concentration at 3× standard deviation above a background mean, which was determined in a sample that had not been supplemented with antigen. In analogy to this, the limit of quantification (LOQ) was determined at 10× standard deviation above background mean. Heat maps were based on hierarchical clustering and Pearson correlation. For comparative analysis, replicated and outlying samples were excluded, and outlier identification was based on Mahalanobis distances calculated from a principle component analysis.² The numbers of remaining samples used are stated in supplemental Table 2. For discovery, p values were calculated for a comparative analysis using a linear model (limFit) of the limma package (28), and standard errors were moderated using a simple empirical Bayes model to compute a moderated t statistic for each comparison and for each antibody. For validation, profiles were age-normalized with a linear regression model to calculate residual values, and Mann-Whitney tests were performed revealing p values. The dependence between profiles and clinical PSA values was calculated using Spearman’s rank correlation (ρ).

RESULTS

Concept for Multiplex Analysis of Plasma or Serum—The concept for high throughput systematic protein profiling using antibody suspension bead arrays is shown in Fig. 1. Antibodies are normalized to the same concentration, coupled to

² B. S. Kato and J. M. Schwenk et al., unpublished results.

Fig. 1. Schematic view of bead array assay. Samples (plasma, serum, urine, etc.) are distributed into microtiter plates (1) following a defined, randomized plate layout. Activated biotin (2; 2) is added to modify the protein content of the samples (3) followed by quenching of remaining labeling reactivity. Antibodies are coupled onto beads with distinct color codes (4), and bead arrays in suspension are created. Labeled samples are heat-treated (5) after being diluted in assay buffer, and without removal of biotin excess, beads and samples are combined and incubated (6). Proteins that have not been captured by the antibodies are washed away (7), and fluorescently labeled streptavidin is added (8) to bind to the biotinylated target protein. The color-coded beads are identified via a green laser, and the concurrently emitted reporter fluorescence, excited by a red laser, allows for the assignment of intensity values (9).
a batch of 384, and 86% of the tested antibodies did bind selectively to the respective antigen (29). Nearly 11,000 (58%) of these antibodies have a concentration of more than 50 μg/ml after affinity capture from the polyclonal sera. Antibody solutions with a concentration less than 50 μg/ml were shown to yield significantly lower fractions of proteins to be detected above the noise of the assay (data not shown). A further selection was based on Western blot analysis, harvesting only antibodies that showed a single band at the expected protein molecular weight (+20%) tested with a single plasma sample, and the resulting list contained 531 antibodies. To match the number of antibodies with the available bead color codes, 96 antibodies from that list were further selected for the study based on their signal intensity levels on the protein arrays, their overall performance in Western blot analysis, and their concentration. Subsequently, these antibodies were immobilized on beads to create a bead array (supplemental Table 1).

Antigen Retrieval Efficiency Using Different Temperatures—The effect of thermal sample treatment on antigen retrieval with different temperatures was explored. Heating undiluted samples at temperatures above 56 °C caused the plasma to clump, presumably due to denaturation and precipitation of many proteins in the plasma (data not shown). Several buffers previously involved in the development of the beads arrays (19) were therefore tested, and a buffer consisting of polyvinyl alcohol, polyvinylpyrrolidone, and casein was selected for the epitope retrieval experiment (see “Material and Methods” for details). One plasma sample was processed in triplicates and analyzed using the 96 antibodies in the assay described above with heat treatment ranging from 23 to 96 °C. For each antibody, absolute signal intensities determined at different temperature levels were compared with the absolute signal intensities at 23 °C (Fig 2A), and the number of intensity increases peaked at 56 and 72 °C. To display alternations between no heat treatment and heat treatment, the intensity values for the antibodies were normalized to compare protein profiles in relation to 23 °C (Fig. 2A). A summary of the trend in intensity changes are shown in Fig. 2B. Plasma treated at 56 and 72 °C showed a similar pattern ($R^2 = 0.92$) and in both, about 40% of the antibody-defined protein profiles revealed positive intensity fold changes, and about 40% displayed negative intensity fold changes (Fig. 2B). Alterations from 45 °C were less prominent but similar to those of the temperatures described above ($R^2 > 0.75$). The results for heat treatment at 96 °C were not advantageous, probably due to a heat-driven denaturation of the probe. These results show that protein profiles are temperature-dependent and

Fig. 2. Effect of heat treatment on protein profiles. Ninety-six antibodies were used to analyze samples treated at temperature levels between 23 and 96 °C in triplicates. A, the number of antibodies that showed increased (solid line) and decreased signal (dotted line) are shown as compared with the values at 23 °C. B, the results for each antibody are shown, and the intensity -fold changes ($\log_2$) was plotted relative to alterations in comparison with 23 °C.
suggest that epitope accessibility can be altered and improved via heat treatment.

Limit of Detection at Different Antigen Retrieval Temperatures—An important issue for biomarker discovery approaches is the sensitivity of the assay. To elucidate the effect of heat on relative limit of detections, anti-PSA antibodies produced in mouse (8A6) and goat (1344) were used to analyze PSA-spiked plasma treated at 23 and 72 °C in parallel. The results are shown in Fig. 3A with the observation that the overall signal intensity decreased when samples were treated at 72 °C as compared with 23 °C. When LOD values were calculated for both temperatures for the anti-PSA antibody 1344, a 3-fold change down to 3 ng/ml was noticed with increased temperature, whereas LOQ values of 27 (23 °C) and 33 ng/ml (72 °C) were calculated. The LOD determined with the anti-PSA antibody 8A6 was altered by a factor of 8 down to 1 ng/ml PSA after heat treatment to 72 °C with LOQ values of 32 (23 °C) and 18 ng/ml (72 °C). During this analysis of plasma spiked with recombinant PSA, a reduction in anti-PSA-derived intensity levels and changes in curve shapes were experienced alongside the temperature increase, the intra-assay variability was not affected, and the limit of detection appeared to be improved with the applied calculation method. Interestingly, heat treatment appeared to affect the ability of the antibodies to capture the target protein in an individual manner, suggesting an epitope-specific effect of heat-induced antigen retrieval in plasma.

Analysis of PSA Levels at Different Epitope Retrieval Temperatures—The influence of heat treatment of plasma samples was further investigated with the perspective of discovering proteins associated to disease or disease state. Plasma samples were collected from unidentified men during PSA testing in the routine laboratory and divided into four different groups of about 20 patients in each group based on total PSA values and free-to-total PSA ratios (supplemental Table 2). The probability of having prostate cancer was high in group A.
and low for group B, whereas the remaining two groups, C and D, represented patients in the gray zone. The amount of PSA was determined in the 80 plasma samples using anti-PSA antibodies. The technical reproducibility was assessed via three samples distributed in triplicates across the microtiter plates, and variability levels for the three temperature treatments had coefficients of variation <16%. The signals obtained using the array-based methods at 23 and 72 °C, respectively, were subsequently compared with the PSA value obtained using a routine method used in the clinic (Fig. 3B). The intensity levels determined in samples with low tPSA content were found to be less scattered and to decrease with temperature, and the average correlation appeared to improve from $R^2 = 0.87$ for 23 °C to $R^2 = 0.94$ for 72 °C. When using these antibodies to reflect the different PSA-based patient groupings, significant differences of $p \leq 7 \times 10^{-6}$ (supplemental Table 3) between group A (tPSA, >60 ng/ml) and the remaining patient groups B–D (all tPSA, ≤10 ng/ml) were obtained. After a sample treatment at 72 °C, additional differences could be displayed for group B (tPSA, ≥1 ng/ml) compared with groups C and D (tPSA, ≥5 ng/ml; both differing in free-to-total PSA ratio). This increase in capability to separate tPSA-based sample groups with an elevated retrieval temperature followed the observations that the detection of recombinant PSA appeared to be improved upon heat treatment.

**Biomarker Discovery Using Different Antigen Retrieval Temperatures**—The plasma samples from this patient cohort were subsequently analyzed in a pilot study using the 96 antibodies selected above (supplemental Table 1). Within a few hours, almost 30,000 data points (3 × 96 patient samples) can be obtained from samples treated at epitope retrieval temperatures of 23, 56, and 72 °C. As illustrated by the heat maps in Fig. 4A, different overall patterns were created for each treatment that promoted the potential to identify new contributors in the classification of patient and disease groups. To determine significant differences between different groups and for the temperature treatments, a linear model was applied to calculate $p$ values, and a summary can be found in Fig. 4B. The results demonstrate that proteins with differential protein profiles ($p < 0.01$) were detected at all heat retrieval temperatures. A number of potential biomarker candidates were identified in this discovery cohort (supplemental Table 4), and almost all were found to be significantly different using one of the three retrieval temperatures. The protein profiles for carnosine dipeptidase 1 protein (CNDP1), however, were shown to be significantly different both at 23 and 56 °C antigen retrieval treatment when patient group A was compared with the other three groups, B–D. Box plots showing the distribution of signal intensities for all the patients in the different patient cohorts are shown in Fig. 4B for each of the protein targets. Additionally, the experimental reproducibility was judged by the concordance of protein profiles from two independent experiments, which were performed months apart, that included all antibodies and all samples treated at 56 °C. The reproducibility of profiles was therefore determined by Spearman correlation ($\rho$) and shown for antibodies that displayed statistical significance of $p < 0.01$ in both independent experiments. The latter criteria was fulfilled by anti-CNDP1 ($\rho = 0.83$), anti-orexin receptor type 1 (HRCTR1) ($\rho = 0.90$), and anti-ghrelin (GHRL) ($\rho = 0.79$).

**Technical Validation of Potential Biomarkers**—For three of the discovered potential biomarkers, a technical validation of the results was performed by Western blot analysis. Three individual patient samples from each group were depleted of IgG and albumin and blotted, and three antibodies were applied to investigate the difference of specific bands in individual patients (Fig. 5A). The analysis of HRCTR1, which was found using the 56 °C retrieval temperature, showed a very clear difference between the two groups with a strong band in the expected size (48 kDa) in all three plasma samples from patients in cohort B, whereas much less protein was observed for plasma from cohort A (Fig. 5A). For CNDP1 discovered both at the 23 and 56 °C retrieval temperatures, an interesting pattern was observed. For this protein, tissue and serum isoforms have been suggested (30), and it is predicted to be $N^\alpha$-glycosylated at two asparagines (Asn-322 and Asn-382). Although bands detected at ±190 kDa probably refer to the glycosylated isoform, CNDP1 appeared to be present in similar amounts for all six samples. A second weaker band at ±55 kDa matches the predicted size of 57 kDa of the unglycosylated isoform (Fig. 5A), and bands of this size were differentially present between the two sample groups. The results suggest that the antibody binds exclusively to this form of the dipeptidase enzyme using the suspension bead array, whereas both isoforms are recognized using the Western blot analysis. The results from both assay platforms therefore indicate that the concentration of potentially non-glycosylated isoform differs between the patient cohorts, whereas the glycosylated form does not vary in a significant manner. For the third protein target, transmembrane protein 59-like (TMEM59L), a less clear pattern was observed. Bands in the right size are present (55 kDa), but the relative pattern between the two groups is less evident, although three of the four strongest samples were indeed from group B. In conclusion, the Western blot analysis confirms the majority of findings from the high throughput suspension bead array assay and also adds information regarding modification-specific levels of one of the protein targets.

**Validation of Potential Biomarkers in Independent Patient Cohort**—The markers confirmed by the Western blot analysis, alongside those listed in supplemental Table 4, were further validated to exclude false positive findings made in association to a specific cohort rather than to disease. An independent and different patient cohort of prostate cancer patients from a separate clinical investigator was used. Biobank plasma samples from patients diagnosed with two classes of aggressive phenotype were obtained together with samples.
FIG. 4. Biomarker discovery at different epitope retrieval temperatures. A, the discovery cohort (supplemental Table 2A) was analyzed at the three treatment temperatures, and the patterns obtained in the heat maps visualize the apparent influence of heat treatment on protein profiles. B, from the comparative study of patient groups, antibodies were identified with significantly different protein profiles ($p < 0.01$) in a
from a control group (supplemental Table 2B). Thirty samples from each of the three groups were assayed with a 56 °C treatment. For these results, the association between protein profiles and age was taken into account, and a clear trend showed that many markers discovered as significantly different in the discovery cohort were not verified in the validation cohort. However, profiles determined for CNDP1 were confirmed to be significantly lower ($p = 0.026$) in aggressive cases as compared with less aggressive cases as shown in Fig. 5B. This was opposite to anti-PSA antibody (1344) for which protein profiles were associated with more aggressive disease. Profiles obtained for CNDP1 and PSA were found not to correlate ($p = -0.2$). Western blot analysis revealed differences in CNDP1 profiles that were in concordance with the results obtained above and based on no detectable bands at ±55 kDa in samples of more aggressive cases (data not shown).

**DISCUSSION**

Here, we show that antibody-based profiling using heat-induced antigen retrieval in combination with a suspension bead array approach allows for sensitive protein profiling in human plasma in a systematic manner. A suspension bead array was used in this work, but it is also possible to use other heat treatment-dependent manner as summarized in supplemental Table 4. To highlight some of the results, the findings from a comparison of patient groups with very high (group A) and normal (group B) PSA values were analyzed using Western blot analysis. For anti-HCRTR1 (HPA014018; top), differences in protein abundance appeared for a ±50-kDa variant of HCRTR1. With anti-CNDP1 (HPA008933; middle), evenly strong bands were observed at a molecular mass of ±190 kDa, matching a glycosylated form of CNDP1. Weaker bands found at ±55 kDa most likely represent the non-glycosylated isoform. Plasma analyzed with anti-TMEM59L (HPA010661; bottom), a glycoprotein with an unmodified isoform of 37 kDa, revealed bands at ±55 kDa, and among the four stronger bands, three were from samples of group B. A second and independent set of samples composed of patients diagnosed with different phenotypes of prostate cancer and controls was studied. Profiles were age-normalized and appeared to be lower for anti-CNDP1 when aggressive and less aggressive cases were compared. This was contrast to profiles from an antibody targeting PSA (1344), MFI, median fluorescence intensity; AU, arbitrary units. For each patient group the box-and-whisker plots represent intensities within lower and upper quantile (box), the median (horizontal line in box), percentiles of 5% and 95% (whiskers), and outliers (open circles).
array formats, such as planar microarrays with spotted antibodies (24) or microfluidic devices with antibodies distributed to microchannels (31). However, the suspension bead array format combines a need for low plasma or serum sample volumes with a flexible assay in which individual antibodies can be exchanged simply by combining different sets of antibody-coupled beads. The method described here was performed with both monoclonal and polyclonal antibodies, and there are no reasons why other affinity reagents, such as aptamers, would not work after immobilization to the color-coded beads. We estimate that \(\sim 10 \mu l\) of plasma or serum from an individual patient sample is needed to perform assays for the analysis of 10,000 target proteins. This makes this assay very suitable for screening biobank material because only minute amounts of precious material is required to allow for individual analysis of thousands of protein targets using specific antibodies.

For the first time, we have performed a systematic study of antigen retrieval for antibody-based plasma analysis. The choice of heat, instead of enzymes, detergents, or pH often used for antigen retrieval in immunohistochemistry assays (32), makes the protocol easy to incorporate into automated procedures. As expected, various antibodies/protein targets respond differently to the heat-induced antigen retrieval, possibly due to a combination of many factors, such as the nature (linear or conformational) and accessibility of the epitope, the solubility of the protein target at different temperatures, etc. However, as shown here, many protein targets benefit from antigen retrieval at 56 or 72 °C. For the anti-PSA binders used, the performance also improved with the heat treatment with regard to relative detection limit and the possibility to resolve differences for PSA levels between 1 and 4 ng/ml. This also implies that other, potentially disease-related proteins with concentrations below 1 ng/ml will remain challenging to detect using the combination of heat-induced epitope retrieval and direct labeling. It would thus also be interesting to evaluate the described method on novel readout systems, such as single molecule arrays (33). After heat treating the samples, it was also observed that the intensity values of the different anti-PSA antibodies did not increase for samples lacking spiked PSA. This was similar for clinical samples with low intrinsic PSA levels where signal fluctuation was reduced. With regard to specificity, this could demonstrate that in these cases unspecific binding is not increasing due to heat treatment.

The dynamic range of protein concentrations found in plasma and serum is very large, spanning at least 9 orders of magnitude (3). Normally, one would use a sandwich assay to allow sensitive assays in the presence of much more abundant serum proteins, but here we show that a single antibody per protein target can be enough to reach proteins with low ng/ml concentrations. We have recently investigated other recombinant human proteins and various antibodies, and the limits of detection based on these assays were also found to be in the lower ng/ml range.\(^3\) It should be possible to increase the sensitivity of the method even further by depleting the most abundant serum or plasma proteins, although the procedure described here is attractive because of its simple preprocessing steps (only biotinylation and heat treatment).

To complement the bead arrays and for evaluation purposes of for example experimental reproducibility or to further narrow down the number of candidates for orthogonal technical validation, planar antibody microarrays can likewise be applied to analyze samples processed as described here (24).

Western blot analysis was used for the initial technical validation. This led to several interesting findings, including the discovery of a potential biomarker consisting of the potentially non-glycosylated protein isoform, whereas the major glycosylated isoform appeared to be similar between the compared groups. It is of course evident that further validation of the potential biomarkers candidates must be completed in larger, independent qualification cohorts, and MS-based methods are required to elaborate on details regarding the glycosylation status of the protein. In the future, it might be interesting to investigate whether the discovered proteins are also differentially expressed in prospective samples of patients not yet diagnosed with prostate cancer. Before any candidate from such affinity-based discovery can be used to draw clinical conclusions, a thorough validation of a potential disease marker is of uttermost importance. Nevertheless, the use of antibodies offers a straightforward validation because the same assay format using the suspension bead array setup also can be used in this case.

The antibody-based profiling described here is an attractive complement to biomarker discovery using other proteomics technologies. Mass spectrometry does not depend on the availability of protein probes, and this technology also allows isoforms and various protein modifications to be analyzed. However, biomarkers found by mass spectrometry must usually be validated with specific antibodies to allow validation in large patient populations and to develop a diagnostic assay suitable for the clinical setting. However, it is of course possible to combine antibody-based protein profiling and mass spectrometry by using immunocapture followed by detailed analysis of the capture protein(s) by mass spectrometry. This might be an excellent choice in the hunt for modification-related biomarkers and might allow for very sensitive determination of low abundance proteins and isoforms.

The throughput achieved with the instrumentation used in this study offers two dimensions of multiplexing to analyze combinations of \(\sim 4 \times 100\) samples with 100 antibodies or vice versa. This means that data from \(\sim 40,000\) assays can be obtained and utilized in a working day, offering a noticeable advantage in terms of sample throughput compared with planar microarrays. Recently, an updated multiplexing system for increased high throughput has been launched (Luminex).

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\(^3\) J. M. Schwenk and H. Langen, unpublished results.
Corps), and with this latest instrumentation, we have been able to utilize 384 antibodies in one single well to profile 384 patient samples. This means that more than 150,000 assays can be performed in a single working day, opening up the possibility for very large scale projects where thousands of biobank samples are studied with thousands of antibodies. In this context, it is important to point out that validated antibodies to proteins corresponding to over 40% of the human genes already exist, and more antibodies are added to the list of public antibodies toward human proteins virtually every day.

In conclusion, we report a method for systematic analysis of biobank samples using multiplexed antibody-based protein profiling, showing that heating plasma prior to the analysis allows, in many cases, for increased performance. The strategy based on bead arrays opens up the possibility for automation using standard laboratory work stations and a proteome-wide analysis of proteins in serum and plasma, enabling biomarker discovery and validation in an undirected and high throughput manner.

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§ To whom correspondence may be addressed. E-mail: jochen.schwenk@scilifelab.se. To whom correspondence may be addressed. E-mail: mathias.uhlen@scilifelab.se.

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