research articles Journal of proteome research

Design of Recombinant Antibody Microarrays for Serum Protein Profiling: Targeting of Complement Proteins

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Received April 11, 2007

Antibody-based microarrays is a novel technology with great promise for high-throughput proteomics. The process of designing high-performing arrays has, however, turned out to be challenging. Here, we have designed the next generation of a human recombinant scFv antibody microarray platform for protein expression profiling of nonfractionated biotinylated human plasma and serum proteomes. The setup, based on black polymer Maxisorb slides interfaced with a fluorescent-based read-out system, was found to provide specific, sensitive (subpicomolar (pM) range) and reproducible means for protein profiling. Further, a chip-to-chip normalization protocol critical for comparing data generated on different chips was devised. Finally, the microarray data were found to correlate well with clinical laboratory data obtained using conventional methods, as demonstrated for a set of medium abundant (micromolar (μM) to nanomolar (nM) range) protein analytes in serum and plasma samples derived from healthy and complement-deficient individuals.

Keywords: Recombinant antibody microarrays • serum proteomics • protein expression profiling • complement proteins

Introduction

Proteomics has become a key discipline within modern biomedicine (disease proteomics) and will provide novel means to perform biomarker discovery for improved and early diagnosis and accelerated drug development, as well as to gain further understanding of disease biology.[1−6] In this context, the human plasma (or serum) proteome is of particular interest, being the primary clinical sample for disease diagnostics.[7−10] The dynamic range (>11 orders of magnitude) and complexity (>10 000 different protein species) of the plasma proteome will, however, place high demands on the technology applied.[8,9,11] While traditional proteomic technologies, such as liquid chromatography or 2-D gel electrophoresis coupled with mass spectrometry, have made significant progress,[7,12] the need for additional technologies for large-scale protein analysis is still obvious.[5,6]

Among the rapidly evolving proteomic technologies, protein microarrays and, in particular, antibody-based microarrays, outline unique possibilities to develop high-throughput, multiplexed, and ultrasensitive assays for global protein expression profiling of complex samples.[13−19] In recent years, several reports have also been published indicating the potential of antibody microarrays for serum or plasma proteome analysis.[1,14,15,19] In these studies, the first generations of small- to medium-scale microarrays have been designed, which are based on intact monoclonal or polyclonal antibodies, targeting mainly high- to medium-abundance analytes.[20−23] Currently, major efforts are under way to further evolve the antibody microarray technology into the high-throughput proteomic research tool needed by the biomedical community.[1,17,19] In this process, several critical areas have been identified,[18,19,24] including (i) content (design, format, and range of specificities), (ii) solid support (biocompatibility and nonspecific background binding), (iii) array format/fabrication, (iv) sample (labeling of complex samples), (v) analytical principle (sensitivity), and (vi) data processing (chip-to-chip normalization).

During recent years, we have focused our efforts on developing recombinant antibody microarrays for high-throughput proteomics.[25−31] To this end, we have used human recombinant single-chain Fv (scFv) antibody fragments, microarray-adapted by design, selected from a large phage display library[32] as probes. Thus, we have instant access to a vast number of high-performing probes displaying an extensive range of specificities.[32,33] By addressing several of the basic technological features, including design and choice of substrate,[26,29] probe

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[#] Decased June 1, 2006.

10.1021/pr070204f CCC: $37.00

PAGE EST: 9.4

format/immobilization,[28,30] sample format/labeling,[25,29] and assay conditions,[29] we have taken the first steps toward designing recombinant antibody microarrays for multiplexed analysis of both low- and high-abundance analytes in complex samples.[29,34]

In this study, we have developed the next generation of our recombinant scFv antibody microarrays for differential protein expression profiling of nonfractionated serum and plasma samples. The setup, based on black polymer Maxisorb slides interfaced with a fluorescent-based imaging system, was evaluated and optimized with respect to sample labeling, sensitivity, specificity, reproducibility, and data handling. The technology platform was validated by comparing the observed microarray data with data obtained using conventional analytical methods, targeting eight complement proteins, including C1q, C1s, C3, C4, C5, C1 esterase inhibitor (C1-INH), factor B, and properdin, in a set of clinically well-characterized serum and plasma samples from healthy and complement-deficient individuals.

Materials and Methods

Proteins and Serum/Plasma Samples. Nine human recombinant scFv antibody fragments directed against eight complement proteins, including C1q (clone C1q-4), C1s (clone C1s-8), C3 (clone C3-7), C4 (clone C4-3), C5 (clone C5-12), C1 esterase inhibitor (C1-INH) (clone EI-12), factor B (clone FB-7), and properdin (clone Prop-3), as well as against cholera toxin subunit B (CT) (clone CT-17), were selected from the n-CoDeR library.[32]

Purified human C1q, C3, and Factor B were purchased from Quidel (San Diego, CA). CT, originating from *Vibrio cholerae*, was purchased from Sigma (St. Louis, MO) and Alexa-647-labeled streptavidin from Molecular Probes (Eugene, OR).

A large pool of normal human sera (NS), 4 normal plasma samples (NP1−4), 5 complement-deficient serum samples (DS1−5) (i.e., samples with significantly altered levels of one or several complement proteins), and 4 complement-deficient plasma samples (DP1−4) were obtained from the Department of Clinical, Microbiology and Immunology, Lund University Hospital (Lund, Sweden). A pool of normal sera, rather than individual samples, was adopted, since the NS was used as a validated reference in the daily routine analysis of complement proteins in clinical samples.

In normal human serum, the concentrations of the CFs are estimated to decrease in the order C3 (6500−8100 nM) > Factor B (2100−5500 nM) > C4 (1700−2900 nM) > C1-INH (900−2700 nM) > C1s (360−960) > C1q (150−650 nM) > C5 (350−450 nM) > properdin (90−140 nM). The levels of complement proteins in the complement-deficient serum and plasma samples were determined using electroimmunoassay (rocket immunoelectrophoresis) and/or turbidometry (C3 and C4). The analytical ranges of these methods ranged from 1 to 6% up to 400% of the normal serum concentration of the protein (electroimmunoassay), and 0.05−20 g/L (C3) or 0.01−4 g/L (C4) (turbidometry). The protein concentrations of the serum and plasma samples were determined using a Micro BCA Protein Assay Reagent Kit (Pierce, Rockford, IL).

Production and Purification of scFv Antibodies. All scFvs were produced in 100 mL of *Escherichia coli* cultures and purified from the periplasmic space using affinity chromatography on Ni-NTA agarose (Qiagen, Hilden, Germany). Bound molecules were eluted with 250 mM imidazole, extensively dialyzed against PBS, and stored at 4 °C until further use. The protein concentration (average concentration 375 μg/mL, range 80−1000 μg/mL) was determined using the Micro BCA assay (Pierce). The integrity and degree of purity (>95%) of the scFv antibodies were evaluated by 10% SDS-PAGE (Invitrogen, Carlsbad, CA).

Biotinylation of Serum and Plasma Samples. All serum and plasma samples were biotinylated using EZ-Link Sulfo-NHS-LC-Biotin (Pierce), since we have recently shown that biotin was the preferred choice of tag for labeling complex samples for sensitive antibody microarray analsysis.[29] First, 50 μL serum aliquots were centrifuged at 16 000*g* for 20 min at 4 °C and diluted 1:45 in PBS, resulting in a concentration of about 2 mg/mL. Next, an internal standard protein, CT (11.6 kDa), that could be used for chip-to-chip normalization was spiked into the samples. CT was selected as standard protein since it is not normally present in serum and plasma, and a high-performing anti-CT scFv was available. Three different concentrations of the spike-in protein, 28, 280, and 2800 nM, estimated to give reasonable signals, were evaluated. The samples were then biotinylated by adding sulfo-NHS-biotin for 2 h on ice, with careful mixing every 20 min. Seven different molar ratios of biotin/protein were evaluated, including 0.7:1, 1.7:1, 5:1, 15:1, 30:1, 60:1, and 150:1. The molar ratio was calculated assuming an average molecular weight of 50 kDa for the serum proteins.[8] Unreacted biotin was removed by dialysis against PBS for 72 h using a 3.5 kDa MWCO dialysis membrane (Spectrum Laboratories, Rancho Dominguez, CA). The samples were aliquoted and stored at −20 °C until further use.

Production and Handling of scFv Microarrays. The scFv microarrays were fabricated using a noncontact printer (Biochip Arrayer1, Perkin-Elmer Life & Analytical Sciences, Wellesley, MA), which deposits about 330 pL/drop using piezo technology. The scFv antibodies were arrayed by spotting 2 drops at each position, and the first drop was allowed to dry before the second drop was dispensed. The antibodies were spotted onto black polymer MaxiSorp microarray slides (NUNC A/S, Roskilde, Denmark),[29] resulting in an average of 8 fmol scFv per spot. Eight replicates of each scFv clone were arrayed in the same row to ensure adequate statistics. The arrays were blocked with 5% (w/v) fat-free milk powder in PBS for 1 h. All incubations were conducted in a humidity chamber at room temperature for 1 h. The arrays were washed four times with 60 μL of 0.05% (v/v) Tween-20 in PBS (PBS−T). Next, 60 μL of biotinylated serum or plasma sample, diluted 1:10 (resulting in a final dilution of 1:450) in 1% (w/v) fat-free milk powder and 1% (v/v) Tween-20 in PBS (PBS−MT) was added. The arrays were washed four times with 60 μL of PBS−T and subsequently incubated with 60 μL of 1 μg/mL Alexa-647-labeled streptavidin diluted in PBST−MT. Finally, the arrays were washed four times with PBS−T and dried under a stream of nitrogen gas.

Analysis and Normalization of scFv Microarrays. The arrays were scanned using a confocal microarray scanner (ScanArray Express, Perkin-Elmer Life & Analytical Sciences) with 5 μm resolution. The ScanArray Express software V2.0 (Perkin-Elmer Life & Analytical Sciences) was used to quantitate the intensity of each spot using the fixed circle method. The local background was subtracted, and to compensate for possible local defects, the two highest and the two lowest replicates were automatically excluded. Each data point represents the mean value of the remaining four replicates.

Two different normalization methods were evaluated in this study: (i) normalization based on an internal standard protein

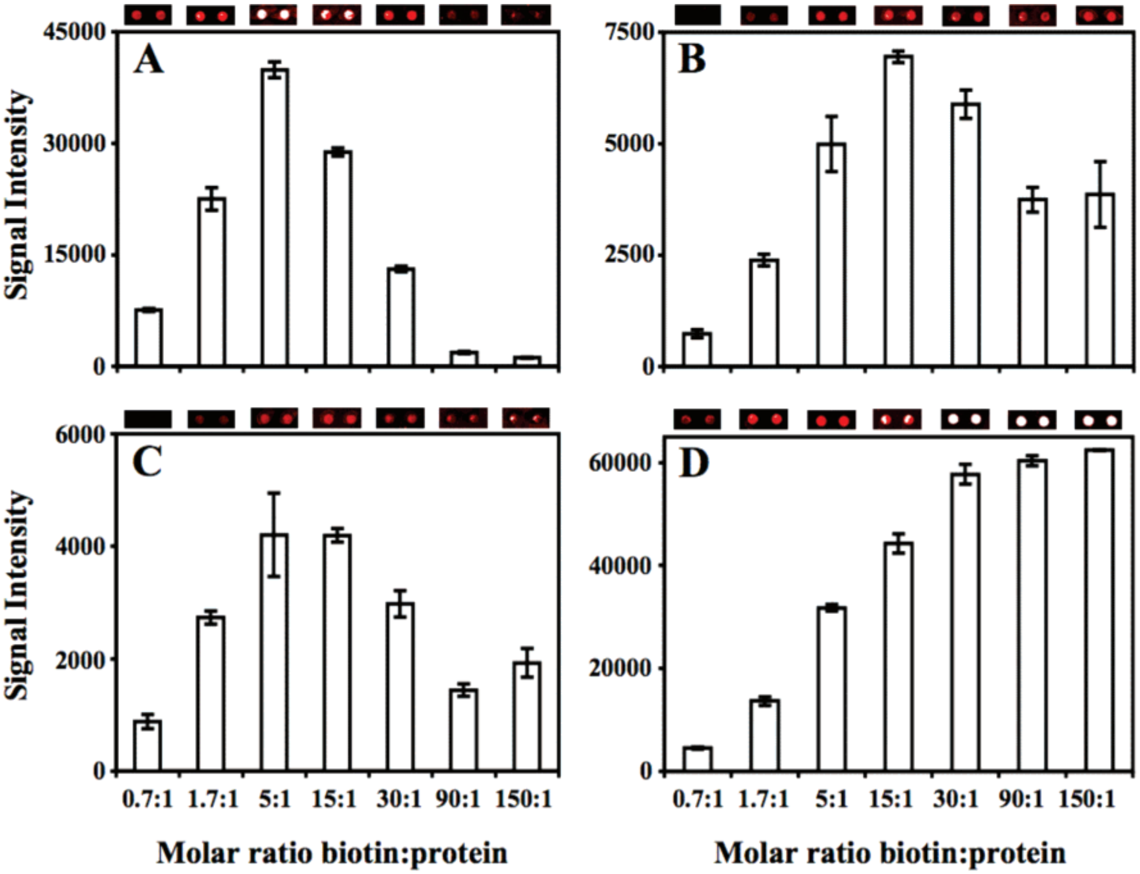


Figure 1. Influence of the degree of biotinylation on serum protein expression profiling using recombinant antibody microarrays. Normal serum was labeled with biotin at molar ratios of biotin/protein ranging from 0.7:1 to 150:1, and the samples were then analyzed on a 9-recombinant antibody microarray, targeting eight complement proteins (C1q, C1s, C3, C4, C5, C1-INH, factor B, and properdin), as well as the spike-in protein (cholera toxin subunit b (CT). Representative results, illustrated by those obtained for (A) C1q, (B) C5, (C) C4, and (D) CT are shown, and the matching microarray images are included.

(CT) spiked into the sera/plasma prior to the labeling and (ii) normalization based on a single analyte (C1q or C3) for which the serum or plasma concentrations had been determined using electroimmunoassay.

In the spike-in approach, the normalization factor, N_i, was calculated for each sample (i) by the formula $N_i = S_{(CT17)}/\mu_{(CT17)}$, were $S_{(CT17)}$ was the signal intensity from the anti-CT scFv for each sample (i), and $\mu_{(CT17)}$ was the average signal intensity from all the samples. The anti-CT scFv was spotted in a serial dilution, from 1 to 8 fmol, and only signals from nonsaturated spots within the linear range of the dilution series were used. Chip-to-chip normalization was then performed by dividing the signal data from each sample with the normalization factor.

The single analyte normalization, based on either C1q or C3, was done by calculating a normalization factor based on the formula $N_i = (S_i/S_\mu)/(C_i/C_\mu)$, were S_i was the signal intensity for C1q or C3 in each sample (i), S_μ was the signal intensity for C1q or C3 averaged over all samples, C_i was the concentration of C1q or C3 in each sample (i), and C_μ was the concentration of C1q or C3 averaged over all samples. Chip-to-chip normalization was then performed by dividing the signal data from each sample with the normalization factor.

Specificity and Sensitivity Test. To evaluate the specificity of the arrayed anti-CF scFvs, blocking experiments were carried out. Briefly, pure and unlabeled CFs, including FB, C3, and C1q, were spiked into the biotinylated (45:1) normal sera (NS), and the observed signal intensities were compared to those of unspiked NS sample. The unlabeled analytes were separately spiked into NS at a final (clinically relevant) concentration of 1 and 10 μg/mL (FB and C1q) or 5 and 50 μg/mL (C3).

The limit of detection (LOD) for the arrayed anti-CF scFv antibodies was measured by serial titration (1:450, 1:900, 1:2250, 1:4500, 1:22500, 1:45000, 1:225000, and 1:450000) of biotinylated NS. The LOD was defined as the signal intensity of the negative control plus 2 standard deviations, and a two-tailed t test was used to determine whether the observed signals were significantly above the limit of detection ($p < 0.05$).

Results

Sample Format: Labeling and Normalization Standards. In order to optimize the assay sensitivity, the labeling of serum and plasma proteomes was initially evaluated (Figure 1). To this end, a pool of human NS was labeled at a molar ratio of biotin to protein ranging from 0.7:1 to 150:1. Next, the samples were analyzed on a 9-recombinant antibody microarray, targeting eight complement proteins (C1q, C1s, C3, C4, C5, C1-INH, factor B, and properdin), as well as the spike-in protein Cholera toxin subunit B (CT). Representative results, illustrated by four of the analytes (C1q, C4, C5, and CT) are shown in Figure 1. The results showed that 2 of 9 analytes (C1q and properdin) (Figure 1A) gave highest signal intensities at a 5:1 ration, while 6 of 9 peaked at 15:1 (C1s, C3, C4, C5, C1-INH, and Factor B) (Figure 1B,C), and 1 of 9 at ≥30:1 (CT) (Figure 1D). At high labeling ratios, reduced signal intensities were thus observed,

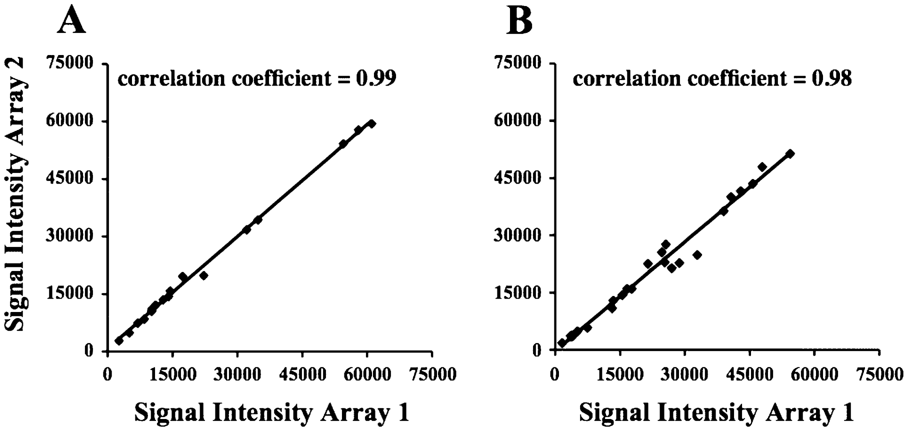


Figure 2. Reproducibility of the antibody microarray analysis. Biotinylated (15:1) normal serum was repeatedly analyzed. (A) Inter-array reproducibility, i.e., the same batch of biotinylated serum analyzed on different microarray slides. (B) Inter-array/-labeling reproducibility, i.e., different batches of a biotinylated (45:1) serum analyzed on different microarray slides. The average correlation coefficient, r^2, is given.

indicating lowered immunoreactivity, that is, epitope masking. On the basis of these results, a molar ratio of biotin to protein at 15:1 was selected and used throughout the study.

Prior to labeling, all serum and plasma samples were spiked with an internal standard protein (CT) to be used for chip-to-chip normalization. Next, the concentration of the spike-in protein was evaluated in order to give adequate signal intensities over a wide range of scanner settings. NS was spiked with CT at three concentrations, 28, 280, or 2800 nM, biotinylated at a ratio of 15:1, and analyzed on an anti-CT microarray containing a serial dilution of the anti-CT probe (0.5−8 fmol). The results showed that a 280 nM concentration of the spike-in protein was sufficient to obtain adequate microarray signals over a broad range of scanner settings (data not shown). Thus, all serum and plasma samples were subsequently spiked with 280 nM CT prior to the labeling process.

Assay Reproducibility. The assay reproducibility was evaluated by analyzing the (i) inter-spot reproducibility, that is, the reproducibility of the eight replicate spots, (ii) inter-array reproducibility, that is, the same batch of biotinylated serum sample analyzed on different microarray slides, as well as the (iii) inter-array/labeling reproducibility, that is, different batches of a biotinylated serum sample analyzed on different microarray slides. In the case of the intra-spot reproducibility, the average coefficient of variation (CV) was found to be <3% (range 1−4%) based on all microarray slides ($n > 30$) analyzed. The different antigen−antibody pairs were found to display similar reproducibility features. Further, the inter-array reproducibility was found to be high, displaying an average correlation coefficient (r^2) of 0.99 (Figure 2A). Similarly, the inter-array/labeling reproducibility was also found to be high, with an average correlation coefficient (r^2) of 0.98 (Figure 2B). Taken together, the results demonstrated a high reproducibility when analyzing complex clinically relevant proteomes.

Assay Specificity and Sensitivity. To evaluate the specificity of the recombinant scFv microarrays, blocking experiments were performed. Biotinylated NS was spiked with unlabeled complement proteins (factor B, C3, or C1q) at two different clinically relevant concentrations (1 and 10 μg/mL or 5 and 50 μg/mL), and the recorded signal intensities were compared with those observed for the non-spiked NS sample (Figure 3). The results showed that the signal intensities dropped 30−57% in a concentration-dependent manner for the anti-factor B probe (Figure 3A). Similarly, the reduction in signal intensities was 32−62% for the anti-C3 probe (Figure 3B), and 81−93% for the anti-C1q probe (Figure 3C). No cross-inhibition was observed for any of the antigen−antibody pairs (data not shown). In all three cases, the signal intensities, thus, decreased in a specific and dose-dependent manner demonstrating the specificity of the arrayed anti-complement protein probes.

Next, the sensitivity of the microarray setup was examined. The LOD was determined by analyzing a serial dilution of biotinylated NS (Figure 4A). Representative results, illustrated by the titration curve observed for the C1q assay, is shown in Figure 4B. The LODs were found to decrease in the order of C1q (0.35 pM) < factor B (4.7 pM) < C5 (8.4 pM) < properdin (10 pM) < C3 (13 pM) < C1s (76 pM) < C4 (210 pM) < C1-INH (530 pM) (Figure 4A). Taken together, the sensitivity of our recombinant antibody microarray technology platform was found to be in the low-to-mid picomolar (pM) range.

Chip-to-Chip Normalization. To be able to compare microarray data generated on different arrays, a chip-to-chip normalization is required to adjust for any nonbiological variations introduced during the assay. Here, we compared and evaluated two main chip-to-chip normalization methods, including (i) normalization based on an internal standard protein (CT) spiked into the samples prior to the labeling and (ii) normalization based on a single serum protein analyte (C1q or C3) of known concentration, as determined using electroimmunoassay. To this end, 6 serum samples and 8 plasma samples were analyzed on our recombinant, anti-complement protein, antibody microarrays.

First, the normalization factor was determined for each sample using both normalization methods. In the case of the spike-in approach, the normalization factors were found to be in the range of 0.7−1.8 (serum) and 0.8−1.4 (plasma). In comparison, the factors were 0.2−2.7 (serum) and 0.2−1.8 (plasma) for the single serum analyte approach. The results also showed that the normalization methods gave identical trends in up- or down-corrections of the data sets, although the spike-in method resulted in lower and less variable normalization factors.

The performance of the single serum analyte normalization was then analyzed by comparing the normalized microarray data (signal intensities) obtained for the spiked-in standard protein with the corresponding non-normalized data as well

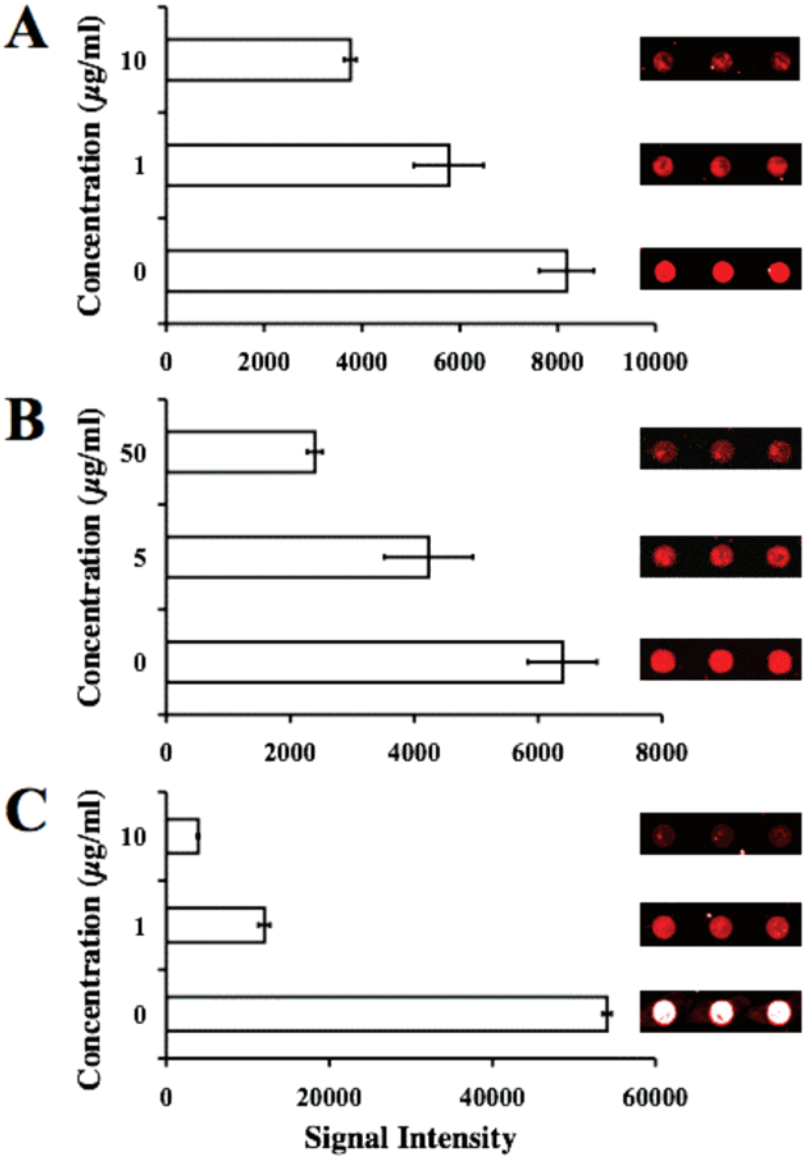


Figure 3. Specificity of the recombinant antibody microarrays. Biotinylated (15:1) normal serum was spiked with pure and unlabed complement proteins, C1q, C3, or Factor B, at two concentrations, and the reductions in microarray signal intensities were measured and compared to those observed for non-spiked NS. (A) Factor B (1 and 10 μg/mL); (B) C3 (5 and 50 μg/mL); and (C) C1q (1 and 10 μg/mL).

A

Analyte	LOD (pM)	K_d (nM)
C1q	0.35	0.2
C1s	76	n.d.
C3	13	500
C4	210	8
C5	8.4	50
C1-INH	530	70
Factor B	4.7	1
Properdin	10	3

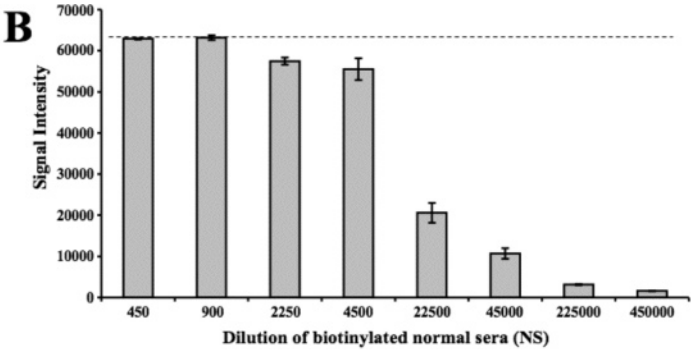


Figure 4. Limit of detection (LOD) for the recombinant antibody microarrays. Serial dilutions of biotinylated (15:1) normal serum was analyzed on our 8 anti-CFs microarrays. (A) LODs for the 8 CFs and the affinity constants (K_d)[32,54] of the antibodies. (B) Serial dilutions of biotinylated serum screened by the anti-C1q scFv. Saturated signals are marked (broken line).

as with the theoretically expected signals (Figure 5A). While the non-normalized data followed the expected titration curve well and displayed a CV < 10%, both the C1q- and C3-normalized data appeared to introduce larger differences with

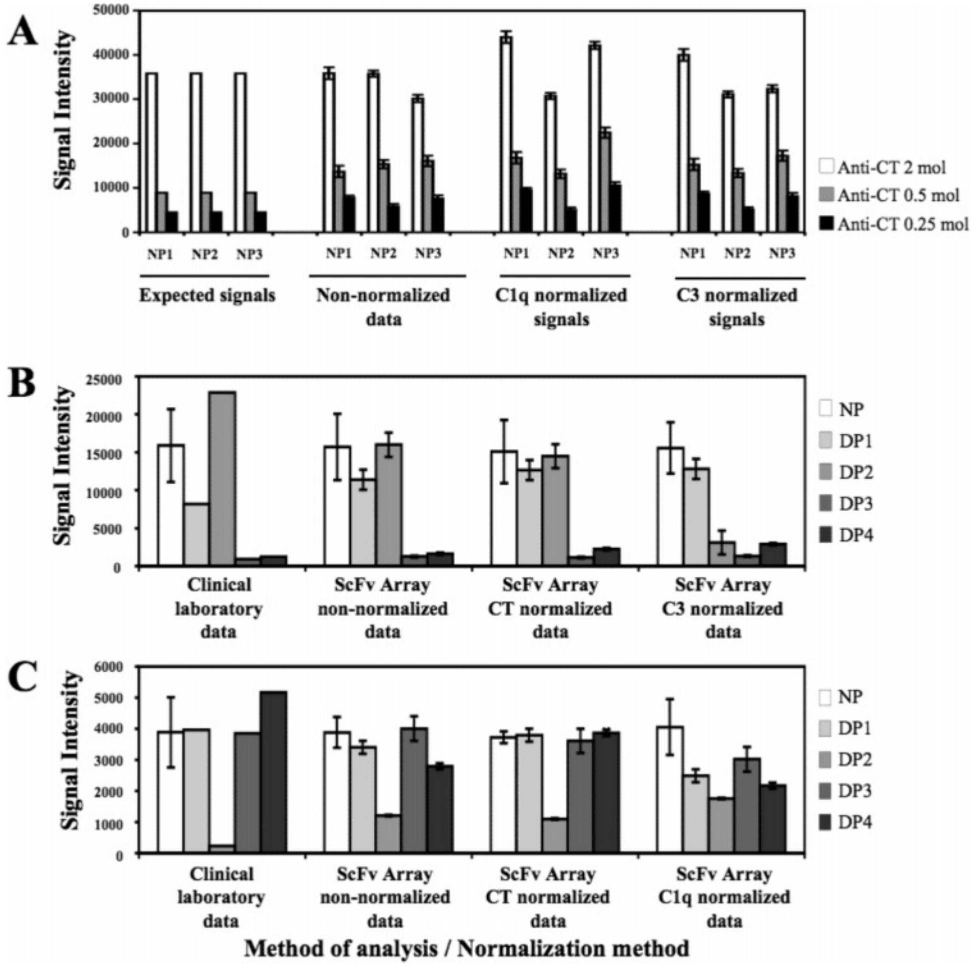


Figure 5. Evaluation of two chip-to-chip normalization methods. (A) Correlation of single serum analyte (C3 or C1q) normalized data with non-normalized data and theoretically expected signals, observed for the internal spiked-in reference protein (CT) in plasma. The theoretical signals were calculated by assuming a linear relationship between the amount of immobilized probe (anti-CT) and the microarray signals. (B) Correlation of single serum analyte (C3) normalized data, spiked-in (CT) normalized data, and non-normalized data with predetermined protein levels, analyzing the expression profile of C1q in plasma. Microarray data for normal plasma (NP) (averaged over four different donors) and 4 deficient plasma samples (denoted DP1−4) are displayed. The clinical levels of C1q were determined using electroimmunoassay. (C) Correlation of single serum analyte (C1q) normalized data, spiked-in (CT) normalized data, and non-normalized data with predetermined protein levels, analyzing the expression profile of C3 in plasma. Microarray data for normal plasma (NP) (averaged over four different donors) and 4 deficient plasma samples (denoted DP1−4) are displayed. The levels of C3 were determined using electroimmunoassay.

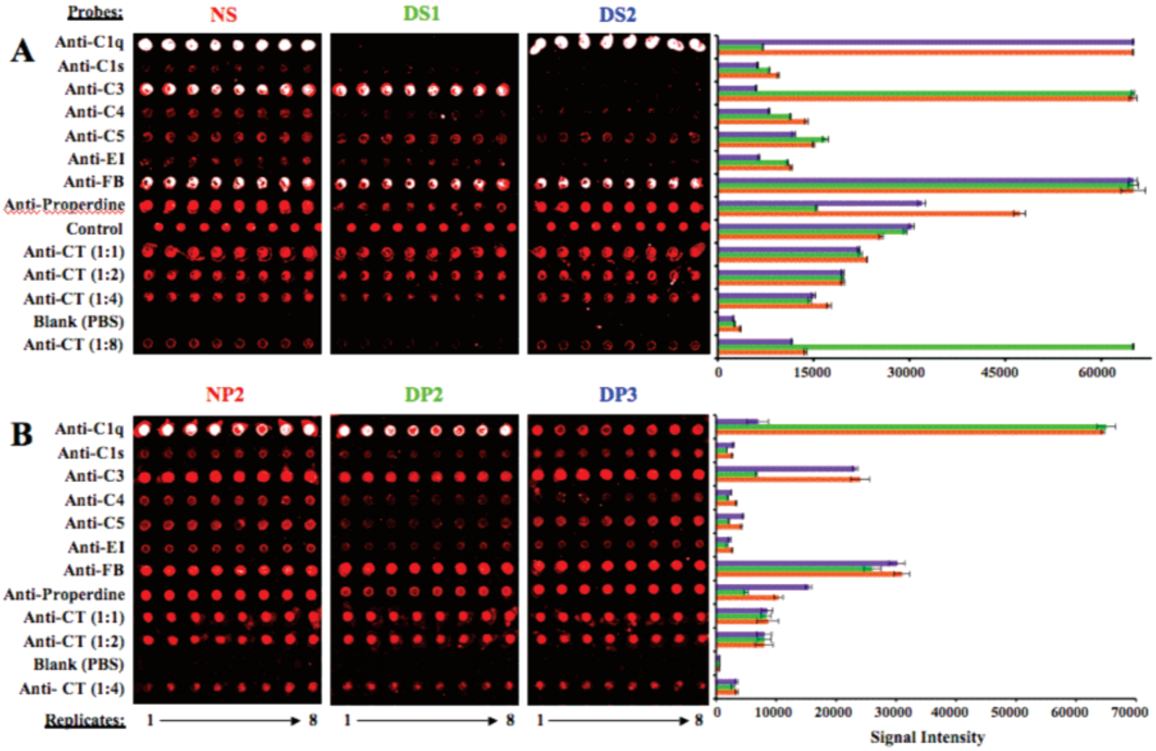
CV values of 27% and 18%, respectively (Figure 5A). Hence, the results implied that single analyte normalization, using either C1q or C3 as reference standard, was not optimal.

Next, we compared the single analyte normalization with the spike-in approach by correlating the normalized microarray data with the known serum and plasma concentrations of the compement proteins. Representative results, illustrated by the profiling of C1q (Figure 5B) and C3 (Figure 5C) in plasma are shown. The spike-in normalization resulted in expression profiles of both C1q and C3 that were similar to those of the non-normalized data and, more importantly, that correlated well with the clinical data (Figure 5B,C). In contrast, the single analyte normalization resulted in expression profiles of both C1q and C3 that displayed large differences compared to the non-normalized data and that also correlated poorly with the clinical data (Figure 5B,C). Similar results were observed looking at the expression profiles of the remaining 6 CFs in both serum and plasma (data not shown). Thus, the results showed that the spike-in normalization was the preferred procedure and was therefore adopted throughout the remaining part of the study.

Differential Serum Protein Profiling. Finally, the optimized scFv microarray technology platform was utilized to perform differential protein profiling of 8 complement proteins in normal versus complement-deficient serum and plasma samples (Figure 6). In total, 6 serum samples and 8 plasma samples were analyzed.

Representative microarray images of normal serum (NS) and deficient serum (DS), as well as normal plasma (NP) and deficient plasma (DP) are shown in Figure 6, panels A and B, respectively. The results showed that distinct and homogeneous spot morphologies with little or no doughnut effects, and very low background signals (nonspecific binding) could be achieved.

Further, we compared the normalized microarray data with the known expression profiles of the CFs, as determined by electroimmunoassay and/or turbidometry. Representative results, illustrated by those obtained for C1q, C3, C5, Factor B,



C

		C1q		C3		C5		FB		Properdine	
		Clinical laboratory data	ScFv Array	Clinical laboratory data	ScFv Array	Clinical laboratory data	ScFv Array	Clinical laboratory data	ScFv Array	Clinical laboratory data	ScFv Array
SERUM	NS	100 %	100 %	100 %	100 %	100 %	100 %	100 %	100 %	100 %	100 %
	DS1	< 2%	1 %	97 %	125 %	88 %	113 %	56 %	81 %	< 5 %	29 %
	DS2	N.D.	114 %	11 %	5 %	118 %	79 %	N.D.	110 %	130 %	68 %
	DS3	93 %	59 %	6 %	15 %	22 %	49 %	N.D.	60 %	121 %	71 %
	DS4	68 %	73 %	107 %	78 %	117 %	88 %	N.D.	89 %	130 %	78 %
	DS5	N.D.	58 %	142 %	59 %	< LOD	19 %	N.D.	73 %	N.D.	58 %
PLASMA	NP	100±27 %	100±30 %	100±30 %	100±8 %	100±25 %	100±16 %	100±20 %	100±21 %	100±20 %	100±10 %
	DP1	53 %	64 %	102 %	105 %	N.D.	92 %	N.D.	90 %	N.D.	91 %
	DP2	148 %	96%	6 %	24 %	N.D.	55 %	N.D.	96 %	15 %	44 %
	DP3	< 6 %	7%	99 %	99 %	N.D.	119 %	N.D.	112 %	N.D.	140 %

Figure 6. Serum protein expression profiling of complement proteins in human serum and plasma samples. The biotinylated (15:1) samples, a large pool of normal human sera (NS), 4 normal plasma samples (NP1−4), 5 complement-deficient serum samples (DS1−5), and 4 complement-deficient plasma samples (DP1−4), were analyzed on a 9-recombinant antibody microarray targeting 8 complement proteins. (A) Microarray images for three representative serum samples, NS, DS1, and DS2, as well as the spike-in normalized microarray signal intensities. (B) Microarray images for three representative plasma samples, NP2, DP2, and DP3, as well as the spike-in normalized microarray data. (C) Correlation of microarray data with known clinical serum and plasma concentrations of the CFs determined using electroimmunoassay. The NP microarray data shown is averaged over four different donors.

and properdin, are displayed in Figure 6C. The results showed that the microarray data correlated well with the expected expression profile of the complement proteins. In more detail, large differences in expression levels (<80% of normal levels) of C1q (DS1, DP3, and DP4), C3 (DS2, DS3, and DP2), C5 (DS3 and DS5), and properdin (DS1 and DP2) could readily be detected (Figure 6C). Medium (approximately 50%) to small (<40%) down-regulated levels (e.g., C1q in DS4) of CFs could also be monitored, whereas the corresponding up-regulations of some analytes (e.g., C1q in DP2) were not detected (Figure 6C). The latter observation could, at least partly, by explained by the fact that the analytes were sensitive to proteolytic degradation and/or we were operating at the high end of the dynamic range of the microarray assay. The latter point could be circumvented by analyzing the serum samples at serial dilutions (data not shown). Taken together, the results showed that we have designed a second generation of high-performing, recombinant antibody microarray technology platform on black polymer Maxisorb slides for differential protein expression profiling of nonfractionated, biotinylated complex proteomes.

Discussion

Affinity protein microarrays based on antibodies is an emerging proteomic technology that will play a major role within disease proteomics, provided that the remaining technologies hurdles can be overcome.[13,15,17−19,24,35] In this study, we have continued to evolve our technology[29] further by addressing some of the remaining technological issues, including sample labeling, sensitivity, reproducibility, and chip-to-chip normalization. Here, we present the next generation of our recombinant antibody microarray technology platform, where these issues have been resolved.

All non-sandwich affinity protein microarray setups have to deal with the challenging task of labeling proteomes.[18,19] While several different dyes are available,[18,19] we have shown that biotin is a preferred candidate for labeling of complex samples, since biotin displays a high biocompatibility, efficient labeling of low-abundance analytes, and low nonspecific binding to blocked solid supports, such as black polymer Maxisorb slides.[29] Here, we have evaluated the degree of labeling in more detail to avoid over-labeling of high- to medium-abundance analytes (micromolar (μM) to nanomolar (nM) range), that is, epitope masking, that could result in impaired probe recognition and reduced assay sensitivity, while still enabling detection of low-abundance analytes (picomolar (pM) to femtomolar (fM) range).[29] Surprisingly, we found the same molar ratio of biotin/protein (15:1) to be optimal for analytes in both the micromolar (μM) to nanomolar (nM) range (Figure 1) as for picomolar (pM) to femtomolar (fM) range.[29] Hence, the results indicated that the individual protein concentration in complex samples was not crucial for the outcome of the labeling. In comparison, other groups have frequently used biotin/protein labeling ratios of around 4:1.[20,22,23] Thus, the results showed that the labeling protocol allowed us to simultaneously target both high- and low-abundance protein analytes in a one-step procedure.

Adopting this labeling protocol, we found the LOD for the medium-abundance complement proteins to be in the low picomolar (pM) range (Figure 4). In agreement, we have found the LOD for low-abundance serum analytes, such as cytokines, to be in the picomolar (pM) to femtomolar (fM) range.[29,30] Hence, this would imply that the sensitivity of our technology platform is within the previously suggested range (attomole level) required for clinical proteomics.[36] Moreover, as previously observed,[25,30] we found no direct correlation between the LOD and either the affinity constant (K_d values) of the scFv antibodies or the serum concentrations of the analytes. This would in turn imply that additional features, such as the labeling efficiency, are affecting the sensitivity. In this context, it is of interest to note that the type of biotin used (Sulfo-NHS-LC-Biotin (www.pierce.com) targeting primary amines (lysines) or ULS-biotin (www.kreatech.com) targeting methionines, histidines, and/or cysteins) could influence the labeling and thereby the assay sensitivity.[29]

The issue of label-dependent versus label-free read-out systems has gained significant interest in recent years (for reviews, see refs 6, 17, 19, 37−40). To date, proof-of-principle has been published for several label-free setups, including SELDI-TOF MS (www.ciphergen.com), MALDI-TOF MS,[31,41] surface plasmon resonance (SPR) and localized SPR[42,43] (www.genoptics-spr.com), nanomechanical cantilevers,[44] and quart crystal microbalance with dissipation monitoring.[45] Albeit promising, these techniques have so far only been used for small prospective arrays, and more developmental work resolving remaining technological issues, for example, sensitivity, will be required before they could become generally applied.

We found the assay reproducibility, illustrated by the inter-spot reproducibility (CV value of 3%, range 1−4%), and inter- and intra-array/-labeling reproducibility (a correlation coefficient (r^2) of 0.98−0.99) (Figure 2), to be equal or better than what have previously been observed for other antibody microarray setups.[21,22] In comparison, many conventional proteomic setups suffer from moderate reproducibility,[6,46] a feature that is not always addressed.[47] Furthermore, the functionality/specificity of arrayed antibodies have in the past been questioned, in particular for setups based on readily available of-the-shelf monoclonal and polyclonal antibodies, that is, reagents that have not been designed for microarray applications.[16,48−50] Here, we showed that our arrayed scFv antibodies, microarray-adapted by design, displayed a high functionality and specificity, as illustrated by blocking experiments and correlation to known clinical levels of the targeted serum protein analytes. Taken together, our optimized setup was found to handle the analysis of complex serum and plasma proteomes well.

To perform differential protein expression profiling analysis and compare data generated on different chips, chip-to-chip normalization must be made to adjust for any nonbiological variations introduced during the assay. To date, very little attention has been placed upon this key issue within the protein microarray community.[18,19] Here, we tested the two best normalization methods recently reported by Haab et al.,[51] including internal standard spike-in and single analyte normalization (Figure 5). As Haab et al., we used a small stable protein, CT (11.6 kDa), to spike the samples with an internal standard of known concentration prior to the labeling. In agreement, we also found this normalization method satisfactory. In contrast, we found the single analyte approach, where the signal intensities from a single protein analyte of á priori known concentration determined by a conventional method was used for normalization, unsatisfactory. In fact, the choice of standard for normalization appeared to be critical, and Haab et al.[51] has also found proteins that displayed a poor performance as single analyte standard. The reason(s) for this impaired behavior remains to be elucidated, but could be related to the stability of the selected protein standard. Compared to Haab and co-workers,[51] we used a much less stable serum protein (C3 or C1q vs IgM) as single analyte standard. Hence, a stable protein resistant to degradation and sample handling (e.g., freezing and thawing) that could be labeled in a reproducible manner would be the ideal candidate as single analyte standard. Further, the simultaneous use of several such standard proteins, present at different concentrations covering a wide dynamic range, may also be advantageous. In future studies, involving larger antibody microarrays, a normalization approach based on “global” normalization similar to what is used for DNA microarrays[52] could be an even better alternative.

Finally, the protein expression profiles of complement proteins, in nonfractionated biotinylated serum and plasma samples, were found to correlate well with the known clinical data (Figure 6). Some minor discrepancies were observed that could be explained by the fact that the complement proteins are sensitive to proteolytic degradation and/or freezing/thawing procedures. The clinical assays were in fact run on fresh samples, whereas our microarray data were generated using aliquots of the same sample that had been stored frozen over extended periods of time. Furthermore, we ran a single assay with one set of fixed parameters compared to eight individually optimized clinical assays. This means that all antibodies will not operate within their optimal (linear) working area, which, to some extent, also could explain the differential (percentual) effects observed in the blocking experiments. Still, by analyzing all the analytes simultaneously, the amount of sample (1 μL) needed was considerably reduced. The dynamic range of a serum and plasma proteins is spanning >11 orders of magnitude,[8,9,11] which might result in the need to analyze samples at serial dilutions. Notably, although we might be operating at the high end of the dynamic range of the setup, we have identified one dilution (1:450) of biotinylated (biotin/protein of 15:1) serum and plasma samples that enabled us to simultaneously target high (micromolar (μM) to nanomolar (nM)) (Figure 6) as well as low (picomolar (pM) to femtomolar (fM)) abundant protein analytes.[29] Moreover, the sample format, for example, serum versus plasma, can also play a crucial role in proteomics, and a recent study has indicated EDTA-plasma to be the preferred choice.[53] While our platform, from a technical point of view, could handle any of these formats, lower and more homogeneous background signals (i.e., nonspecific binding to the solid support) were observed for plasma samples. Further studies using larger sets of samples will be required to elucidate the optimal sample format for antibody microarray-based profiling of blood proteomes.

In summary, this study reports on the next generation of a high-performing recombinant antibody microarray platform based on black polymer Maxisorb slides. This technology platform has allowed multiplexed protein expression profiling of complete, that is, nonfractionated, biotinylated human serum and plasma samples with high reproducibility, demonstrating its potential within (disease) proteomics. In the specific case of complement proteins, complement analysis in clinical practice is mainly used to determine if there is any complement deficiency or aberration due to activation and consumption. The possibility to simultaneously measure several complement proteins in a multiplexed manner is therefore highly relevant and has a potential to improve the use of complement analysis in clinical work.

Abbreviations: CT, Cholera toxin subunit B; C1-INH, C1 esterase inhibitor; DP, deficient plasma; DS, deficient serum; FB, Factor B; LOD, limit of detection; NP, normal plasma; NS, normal serum; scFv, single-chain Fragment variable.

Acknowledgment. This study was supported by grants from the Swedish National Science Council (VR-NT), SSF Strategic Center for Translational Cancer Research (CREATE Health), and Landshövding Per Westlings Foundation.

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PR070204F