

## TECHNICAL BRIEF

# Selectivity analysis of single binder assays used in plasma protein profiling

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The increasing availability of antibodies toward human proteins enables broad explorations of the proteomic landscape in cells, tissues, and body fluids. This includes assays with antibody suspension bead arrays that generate protein profiles of plasma samples by flow cytometer analysis. However, antibody selectivity is context dependent so it is necessary to corroborate on-target detection over off-target binding. To address this, we describe a concept to directly verify interactions from antibody-coupled beads by analysis of their eluates by Western blots and MS. We demonstrate selective antibody binding in complex samples with antibodies toward a set of chosen proteins with different abundance in plasma and serum, and illustrate the need to adjust sample and bead concentrations accordingly. The presented approach will serve as an important tool for resolving differential protein profiles from antibody arrays within plasma biomarker discoveries.

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Affinity-based methods are emerging among today's proteomic tools [1] and there are several projects dedicated to cover the complete proteome with binding reagents [2]. One of these initiatives is the Human Protein Atlas (HPA) [3] that produces, verifies, and applies antibodies to different immunoassays in a systematic and gene-centric manner [4]. One type of these assays is an antibody array based on suspensions of color-coded beads [5] that allows protein profiling of biotinylated serum and plasma proteins in a multiplexed fashion [6–8]. Similar forms of bead arrays have been applied elsewhere to study fractions of cell lysates [9], determining more than 1700 parameters at once [10]. This high throughput potential and the flexibility to create arbitrary panels of antibod-

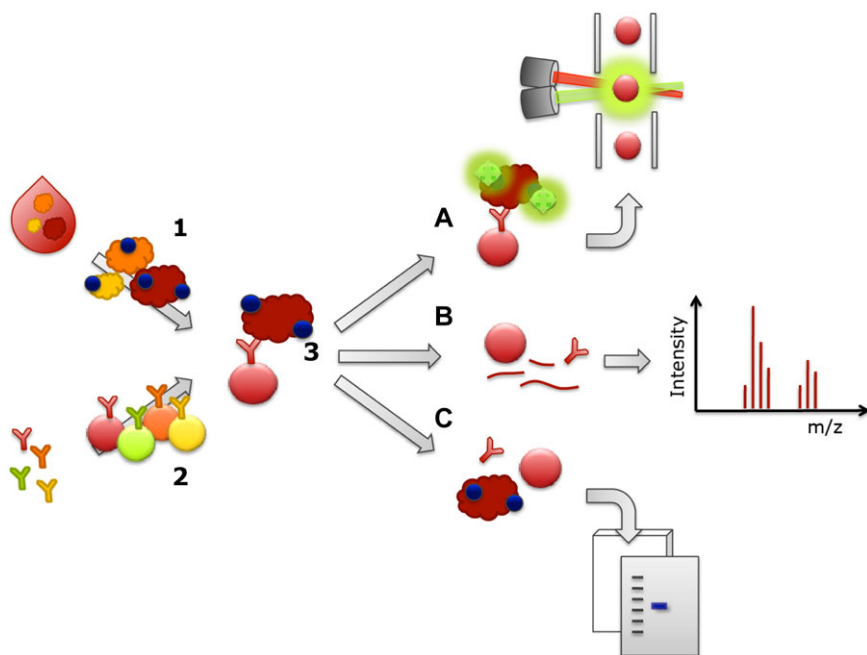
ies is an advantage when using single binder assays compared to the often more sensitive and specific sandwich immunoassays, which though suffer from cross-reactivity of detection antibodies hindering an exploratory assignment [11]. For the verification of antibody array discoveries, it is this far common practice to use orthogonal methods such as Western blotting or ELISA, but since single binder assays can be susceptible to off-target interactions, it often remains unverified which protein(s) were actually captured in such multiplexed analysis. This holds especially true for plasma with its tremendously dynamic range of protein concentrations [12], and the fact that proteins are not locally enriched (as in Western blot or immunohistochemistry) when being detected. To determine which components contribute to signals generated in single binder discovery assays, we describe a procedure that analyzes immunocaptured proteins in parallel by Luminex flow cytometer, Western blot, and MS (Fig. 1). We employed this procedure to explore differential detection of several proteins

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**Abbreviations:** HPA, Human Protein Atlas; NGAL, neutrophil gelatinase-associated lipocalin

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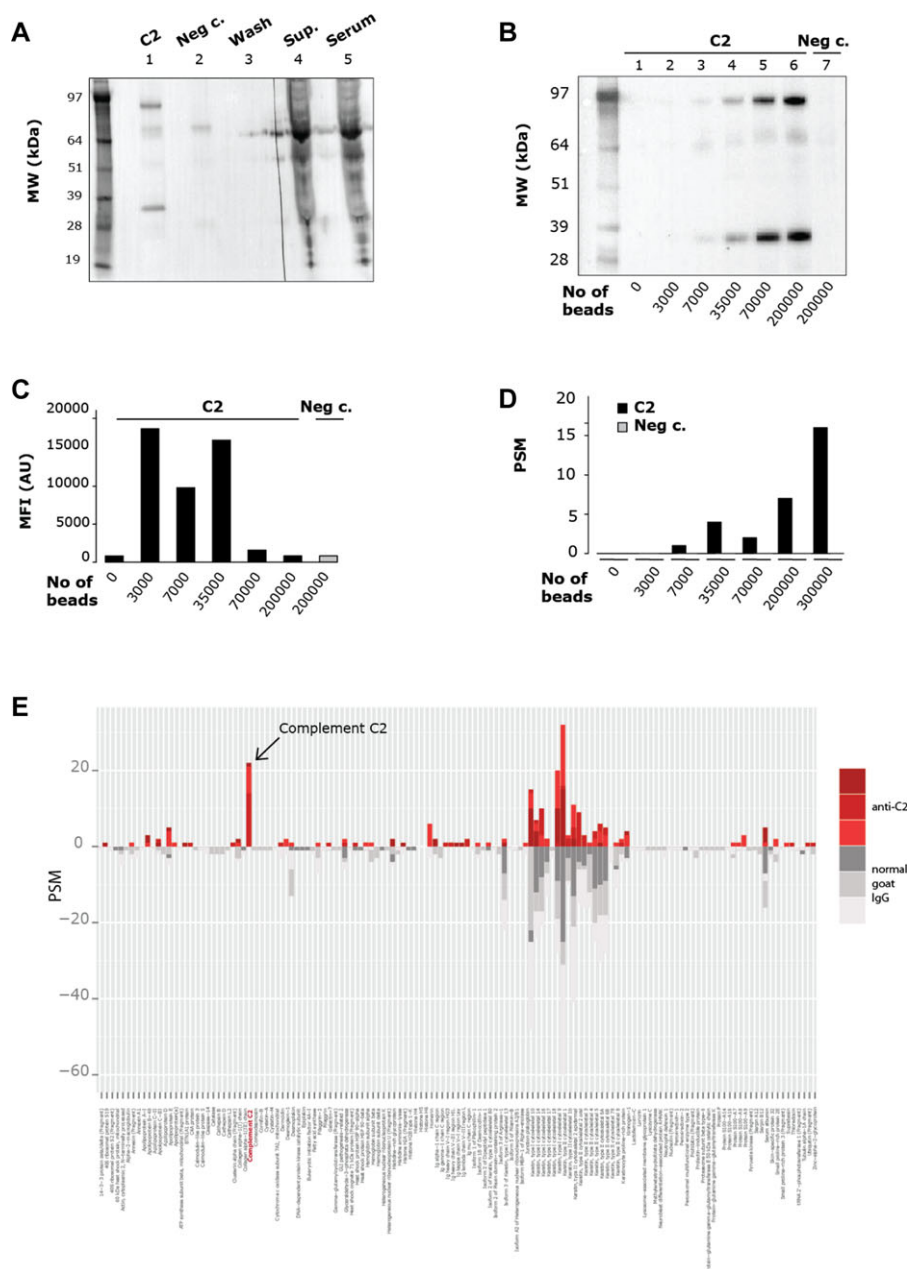


**Figure 1.** Procedure overview. The described procedure allows resolving proteins involved in profiles generated by bead array assays, which is based on parallel analysis of captured proteins by MS or blotting: (1) The protein content of plasma samples is directly labeled with biotin; (2) Antibodies are covalently immobilized on color-coded beads; (3) Beads functionalized with antibodies are incubated with the biotinylated plasma to capture the protein of interest from the solution. The captured protein(s) are then analyzed in parallel using three methods: (A) The relative levels of immunocapture reactions on beads were fluorescence intensity levels derived from a flow cytometer after the addition of a streptavidin fluorophore; (B) The identities of the captured proteins were determined by on bead protein digestion and analysis of peptides using MS; (C) The relative levels, composition, and estimated molecular masses of the immunocaptured protein(s) were evaluated on a Western blot with streptavidin HRP detection.

in biotinylated serum or plasma, also comparing heat-treated with nonheat treated samples. One intention here was to relate information on molecular mass and protein identity to fluorescence intensity levels. The development of this procedure is aimed at achieving a straightforward evaluation of profiles determined during multiplexed discovery analysis of human serum and plasma making use of the experimental flexibilities offered by beads.

To investigate the complementarity of the three methods, we investigated conditions such as numbers of beads and starting volume of crude sample required for several differentially abundant proteins: fibrinogen alpha chain, fibulin-1 (FBLN1), complement component 2 (C2), neutrophil gelatinase-associated lipocalin (NGAL), and chemokine C-C motif ligand 16 (CCL16). Concentrations for the five protein has been estimated to approximate normal concentrations of  $\sim 3$  mg/mL for FG [13],  $\sim 35$   $\mu$ g/mL for FBLN1 [14],  $> 20$   $\mu$ g/mL for C2 [15],  $\sim 60$  ng/mL for NGAL [16], and  $\sim 5$  ng/mL for CCL16 [17]. A bead-based assay was performed for C2 as previously described [8] scaling up sample volume of crude serum to 0.5  $\mu$ L and analyzed in Western blot together with beads coated with immunoglobulins without designated reactivity toward human targets (Fig. 2A). Diluted biotinylated plasma was then incubated with increasing numbers of beads (0–200 000; Fig. 2B) and band at the predicted mass of C2 (83 kDa) was visible when 35 000 beads were incubated with 0.5  $\mu$ L of plasma and a second band at 30 kDa was observed, which may be attributable to the C2 fragment C2b (24 kDa). The analysis of beads in the flow cytometer however showed decreasing signal intensities for  $> 70$  000 of beads (Fig. 2C), reflecting the decreased amount of protein captured per bead. Thus when applying more beads coated with the same antibody to target a less abundant protein, the protein concentra-

tion in solution will be affected resulting in a lower fractional occupancy of binding sites per bead (Fig. 2C). This was extended to repeated analysis in MS. To avoid interference of biotinylated lysine residues during trypsin digestion, non-biotinylated plasma was used (Fig. 2D) which in this case revealed concordant data to analysis of a biotinylated sample (Supporting Information Fig. 1 and Supporting Information Table 2). No peptides originating from C2 were identified when 3000 or less beads were applied, while 1, 4, 2, 6, and 13 peptides were identified for 7000, 35 000, 70 000, 200 000, and 300 000 beads, respectively (Supporting Information Table 3). Approximating this to the number of molecules, there are 1 000 000 binding sites per bead (communication with Luminex Corp) and 0.5  $\mu$ L serum contain 120 amol of C2 protein. This means that at least 70 amol of theoretical antibody-binding sites (35 000 beads) were required for C2 detection in eluates using WB or MS, whereas 0.7 amol (350 beads) were sufficed for analysis of beads with a flow cytometer. In each MS experiment, additional proteins other than C2 were identified. In average, 18 additional proteins were detected per experiment, with a total of 53 different and most noticeably environmental proteins such as keratins. These proteins were though concordant with results from beads coated with normal goat IgG as illustrated in Fig. 2E. C2-peptides were unique only for the anti-C2 beads (Supporting Information Table 4) and confirmed by triplicated analysis. No C2-peptides were found for the normal goat IgG capture. See also Supporting Information Table 3 and Supporting Information MS data for further details. Additional protein targets with similar or higher abundance than C2, such as FBLN1 and FG, were then analyzed when employing around 20 000 beads with 0.5  $\mu$ L of plasma (diluted 1:500). As shown for WB in Supporting Information Fig. 2A a single band was



**Figure 2.** Required number of beads and sample volume. (A) The protein C2 was selectively enriched (lane 1) from biotinylated serum diluted 1:500 and shown on a streptavidin-HRP stained WB. Control beads (lane 2) did not enrich the two bands in lane 1 that match the sizes of C2 (83 kDa) and C2b (24 kDa). Subsequent lanes show the biotinylated protein content from a wash fraction (lane 3), the supernatant after overnight incubation (lane 4), and labeled serum diluted 1:500 (lane 5). (B–D) Increasing number of beads coupled with anti-C2 antibody were incubated with a constant volume of biotinylated serum. Protein captured was analyzed by Western blot (B), flow cytometry (C), and MS (D). In (B) bands of C2 were distinguishable from background in WB with 35 000 beads. In (C) the parallel read-out of protein capture beads by a flow cytometer showed signals with minute amounts of beads. In (D) increasing the number of beads increases the number of identified PSMs (peptides spectrum matches) by MS, revealing a correlation between number of beads and MS signals. (E) Comparative analysis of PSMs obtained in eluates from anti-C2 and normal goat IgG reveal, among several common peptides, C2 peptides for anti-C2 antibody only.

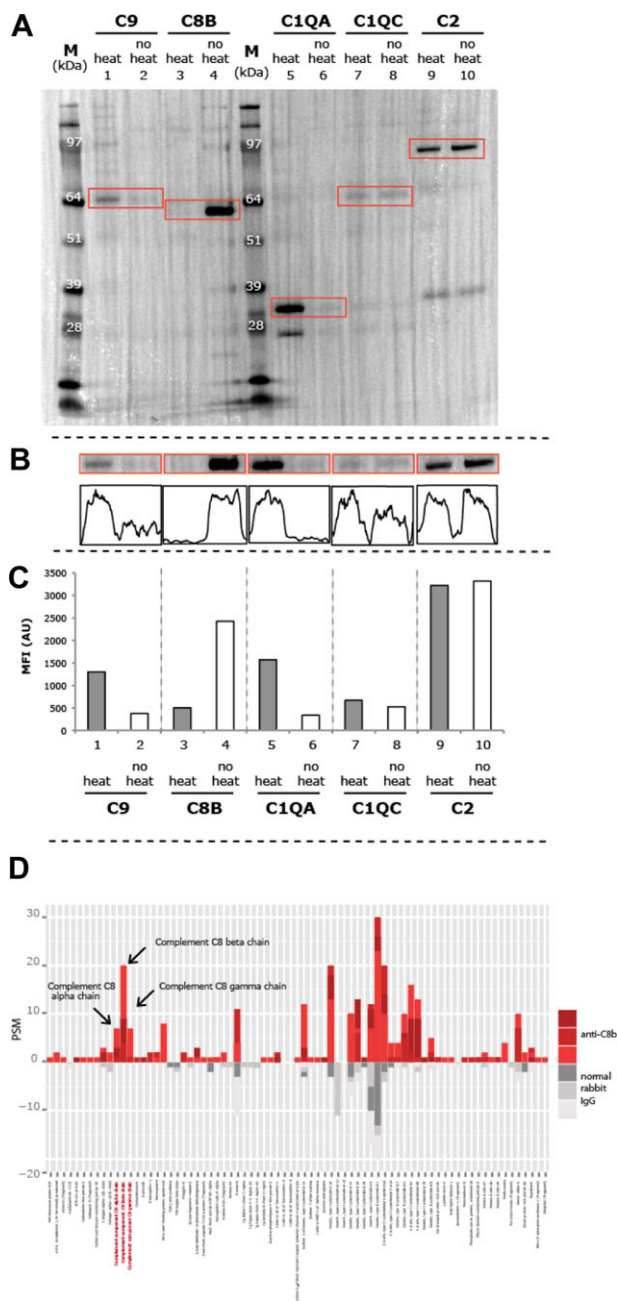
obtained from FBLN1 antibody while anti-fibrinogen alpha chain revealed several bands, both supported by molecular masses predicted for these proteins (see Supporting Information Table 1) and by the blank lane for the negative control. In the analysis of beads with a flow cytometer (Supporting Information Fig. 2B) about one third of the applied beads were counted and resulting in intensity levels that were in agreement to the WB bands. When extending the investigation to the less abundant proteins NGAL and CCL16, an increase of the starting material was used. Indeed, keeping the number of beads to 200 000 and increasing the reaction volume—and thereby the required volume of crude plasma—the amount

of captured and thereby collected protein could be increased. The 200 000 beads coupled with the anti-NGAL antibody were incubated with 0.5, 5, and 25  $\mu$ L of diluted human plasma (Supporting Information Fig. 2C) and in the eluate the protein's band was visualized in WB when applying a biotinylated anti-NGAL antibody for HRP detection. CCL16 was captured using beads carrying antibody HPA042909 and 5, 30, and 100  $\mu$ L of crude plasma were to be detected by anti-CCL16 detection antibody in WB. One clear band was detectable for NGAL with 5  $\mu$ L plasma (Supporting Information Fig. 2D and Supporting Information Table 6), while a band corresponding to CCL16 became detectable when 30  $\mu$ L was applied. In our

hands, the number of beads and sample input needed for detection in WB appeared related to the target concentration as enough molecules need to be collected to be visualized. For the analysis of beads in the flow cytometer, far less beads were needed to exceed the threshold of protein detection. Effectively, the conditions for an analysis of eluted target need to be adjusted for WB and eventually MS analysis, as these measure total amounts of protein carried by the beads rather than targets per bead. This observation is in line with previous works on suspension bead arrays [11] and described in the ambient analyte theory [18]. For the microarray assays used here, target antigens are not depleted from the solution (denoted concentration sensing) and only if the number of beads is increased, the assays leave the ambient analyte area to become mass sensing.

It has been reported that heating blood-derived samples affects proteins of the complement system [19] and also we have previously shown that heat can have enhancing effects on multiplex antibody-based protein detection [8]. To further resolve such findings, we used the proposed verification strategy to evaluate the composition and relative amounts of proteins captured by antibodies targeting C1QA, C1QC, C2, C8B, and C9 in heated and nonheated plasma. A common sample was hereto split for heating at 56°C or incubation at ambient temperature for 30 min. About 200 000 beads per protein target and heat condition were used and split into eight replicate reaction wells, each hosting 0.1 µL crude serum diluted 1:500. For each target and condition, beads from one microtiter plate well were analyzed in the flow cytometer, while the beads from remaining seven wells were pooled for WB analysis. As shown in Fig. 3A–C there is concordance between these two read-out methods as antibodies that reveal profiles with increased fluorescence intensity levels upon heat treatment also showed WB bands of higher intensities and at the predicated molecular mass of the target. Only anti-C1QC capture revealed bands that indicate off-target binding. Even though it remains to be further understood whether denaturation (conformational changes) or changes in protein assemblies (dissolving complexes) altered epitope accessibility, this finding supports that the enhanced or reduced effects of heat treatment detected by single binder assays [6] for these antibodies relates to more or less capture of the intended target protein. MS-analysis of proteins captured by anti-C8B (Fig. 3D), determined that among other proteins concordantly captured by normal goat IgG, the C8-complex including alpha, beta, and gamma chains was captured from a nonheated biotinylated plasma, as seen in Supporting Information Table 4 and Supplementary Information. See also Supplementary Discussion for further reflections on detection of complement proteins.

In conclusion, the presented procedure to analyze antibody-coupled beads used in high-throughput suspension arrays offers an important tool for resolving differently detected serum or plasma protein profiles at an early stage. This strategy will further aid our understanding of antibody and assay performance of discovery driven antibody arrays.



**Figure 3.** Heat-dependent capture profiles. Antibodies raised toward complement proteins C1QA, C1QC, C2, C8B, and C9 were used to study effects of heat on proteins capture performance. (A) One blot revealed biotinylated proteins captured by the five antibodies and (B) major bands per antibody were analyzed from relative band intensities. (C) Corresponding analysis of beads using the flow cytometer showed concordance in heat treatment effects in protein detection. (D) Besides several common peptides, comparative analyses of PSMs (peptides spectrum matches) obtained in MS from eluates from anti-C8 and normal goat IgG reveal peptides of C8 chains alpha, beta, and gamma using anti-C8 antibody.



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