Published in final edited form as:

Anal Biochem. 2011 July 1; 414(1): 99–102. doi:10.1016/j.ab.2011.02.039.

Development of a Fibrinogen-Specific Sandwich ELISA Microarray Assay for Distinguishing Between Blood Plasma and Serum Samples

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Abstract

We have developed a fibrinogen-specific sandwich ELISA microarray assay for use in qualitatively distinguishing between blood plasma and serum samples. Three capture antibodies, 49D2, HPA001900, and F8512, were evaluated in conjunction with 1D6 as the detection antibody. The data show that 49D2 and, to a lesser extent, F8512 successfully identify previously unknown plasma and serum samples based upon a ~28-fold difference in signal intensity between the sample types. This assay has utility in rapidly identifying previously archived clinical samples with incomplete annotation in a high throughput manner prior to proteomics analyses.

Keywords

ELISA; plasma; proteomics; serum

Introduction

Proteomics approaches are widely used to search for clinically relevant biomarkers of disease, and blood plasma and serum are the most commonly used sample types for biomarker discovery [1; 2]. However, the discovery and subsequent validation of candidate protein biomarkers typically requires large numbers of samples; therefore, many studies include samples collected from multiple sites. By now, investigators are well aware of the need for uniformity in all aspects of sample collection when employing proteomics in biomarker discovery studies, in order to minimize confounding factors [3]. However, archived samples exist from many clinical studies in which sample collection protocols were not rigidly controlled or properly recorded. Alternatively, some studies employ assays that are not sensitive to differences in sample type or sample collection. For example, the Diabetes Autoantibody Standardization Program (DASP), a collaboration of the Immunology of Diabetes Society and the U.S. Centers for Disease Control and Prevention, has as its goal the improvement of the performance of assays for islet cell autoantibodies [4; 5]. The measurement of islet cell autoantibodies is commonly performed using solution-based radiobinding assays [6], which are insensitive to differences in sample type. Thus,

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samples collected from patients with recently diagnosed type 1 diabetes and contributed to the DASP may be either serum or plasma. Unfortunately, this represents a confounding factor for potential proteomic analyses of DASP samples for discovery of novel biomarkers of type 1 diabetes, particularly when information regarding sample type is incomplete. Therefore, it is critical to classify samples from this and similar studies as plasma or serum before proteomic analyses are conducted. Traditional methods of distinguishing serum and plasma rely on selectively inducing coagulation in plasma samples [7], and require larger volumes of sample than are typically available from sample archives.

The enzyme-linked immunosorbent assays (ELISA) is an attractive option for differentiating plasma and serum samples [8], as it is sensitive and selective, and can efficiently process large numbers of samples [9]. Furthermore, the biochemistry of fibrinogen processing can be exploited when designing an appropriate sandwich ELISA assay. Intact fibrinogen is present only in plasma. In the conversion of plasma to serum, fibrinogen is proteolytically cleaved by thrombin to form fibrin and fibrinopeptide A [10]. In theory, it should be possible to distinguish between the intact and processed fibrinogen using a sandwich ELISA. This is because the sandwich ELISA utilizes two antibodies to detect distal epitopes on a single protein. The first antibody, called the capture antibody, isolates and concentrates the targeted antigen. The second, or detection antibody, binds to a distal epitope. The detection antibody is tagged to allow for signal quantification, which is directly proportional to the antigen concentration under non-saturating conditions. Therefore, it should be possible to select antibodies that bind on opposite ends of fibrinogen, such that only the uncleaved protein will give a signal in this sandwich ELISA. That is, the use of two antibodies that detect distal portions of the intact fibrinogen protein should be useful for distinguishing between plasma, which has high levels of intact fibrinogen, and serum, which only has trace amounts of intact fibrinogen. Current commercial ELISA kits for measuring fibrinogen are based on polyclonal antibodies that were raised against intact fibrinogen. These antibodies typically detect both the whole fibrinogen molecule found in plasma and the proteolytic products in serum, and therefore cannot be used to distinguish between plasma and serum.

Herein, we describe the development of a sandwich ELISA microarray assay for whole fibrinogen that can be used specifically as a rapid and high-throughput test to distinguish between plasma and serum. This assay can use either of two capture antibodies for intact fibrinogen, as well as a detection antibody to fibrinopeptide A. Fibrinopeptide A was chosen because it is the first cleavage event in the multi-step conversion of fibrinogen to fibrin [11].

Materials and Methods

Antibody Source and Preparation

The fibrinogen antibodies used as capture antibodies in the sandwich ELISA were mouse monoclonal anti-Fibrinopeptide A (49D2; Product No. ab14801; Abcam, Cambridge, MA), rabbit polyclonal anti-Fibrinogen β -chain precursor recombinant protein (HPA001900; Product No. HPA001900; Sigma-Aldrich, St. Louis, MO), and goat polyclonal anti-whole fibrinogen (F8512; Product No. F8512; Sigma-Aldrich). Antibody F8512 was partially purified using a protein A/G Purification Kit (Product No. 20423; Thermo Scientific Pierce Protein Research Products; Rockford, IL), according to Procedure B of the manufacturer's instructions. The detection antibody was mouse monoclonal anti-whole fibrinogen (1D6; Product No. 250310; Abbiotec, San Diego, CA). Prior to its use, we labeled antibody 1D6 with biotin using the Sulfo-NHS-LC-Biotin Kit (Product No. 21435; Thermo Scientific Pierce Protein Research Products), according to the manufacturer's protocol.

Slide Production

The custom manufacture of ELISA microarrays was performed as previously described [12]. Briefly, capture antibodies were printed on aminosilanated glass slides (Erie Scientific, Portsmouth, NH) that were pre-stamped with a hydrophobic barrier to create 16 wells per slide in a 2 by 8 pattern. The spacing of the 8 chips was identical (0.9 cm pitch) to that of a 96-well microtiter plate, allowing for sample transfer using multichannel pipettes. Prior to printing, capture antibodies were suspended in phosphate-buffered saline, pH 7.2 (PBS) at concentrations between 0.5 and 1.0 mg/mL. The antibody solutions were then printed using a non-contact, piezoelectric NanoPlotter NP2 printer (GeSiM, Germany), which prints approximately 400 picoliters per spot, and the spot-to-spot pitch was 400 microns. Each chip also contained several spots of fluorescently labeled, non-immune immunoglobins that were used for orientation, as well as PBS-only blank spots, which were used as negative-control spots, and a capture antibody for green fluorescent protein (GFP). GFP was spiked into all samples at 100 pg/mL, and a biotinylated GFP detection antibody was included in the detection antibody solution, allowing for chip-to-chip evaluations of assay quality and reproducibility based on GFP sandwich ELISA analyses, as previously described [13]. Each reagent was printed onto four spots per chip in a single row. Successful printing of the capture antibodies was confirmed using the Red Reflect option on a ScanArray ExpressHT microarray laser scanner (PerkinElmer, Santa Clara CA). The hydrophobic barrier separating the chips was enhanced using a Pap pen (Bio-Rad, Hercules, CA). After the printed antibodies had adsorbed onto the slide surfaces, the slides were blocked in 1% casein in PBS (Bio-Rad). Slides were then stored at -20°C, with desiccant and under vacuum, until use.

Samples

This work was approved by the Institutional Review Board of the Pacific Northwest National Laboratory. Human plasma and serum samples from the same individual were obtained from Golden West Biologicals, Inc. (Temecula, CA) and received frozen on dry ice. The Golden West samples were used in a preliminary study to optimize the sandwich ELISA protocol. A larger set of human plasma and serum samples from 10 healthy individuals were purchased from Bioreclamation, Inc. (Hicksville, NY) and received frozen on dry ice. The second sample set was used to show specificity and sensitivity of the fibrinogen assay. In the second set, blood was collected from donors both after an overnight fast and again 1 h after the last meal. Samples were randomized and blinded prior to analysis on the ELISA microarray chips; un-blinding of samples was performed after analysis was complete. Prior to ELISA microarray analysis, serum and plasma samples were diluted 500-fold with PBS containing 0.1% casein (BioRad) and 100 pg GFP/mL.

ELISA Microarray Processing

ELISA microarray chips were incubated at room temperature in a closed chamber with saturated humidity using an orbital shaker for gentle mixing; this procedure was used for both the sample and detection antibody incubation steps. A total of 20 µL/chip of each sample (after a 500-fold dilution) was placed on each of three replicate chips, and the chips were incubated overnight. The slides were then washed, and the detection antibody was added at a concentration of 300 ng/mL followed by incubation for 2 h. Signal amplification was performed as previously described [12]. The slides were then imaged with a ScanArray Express HT laser scanner. ScanArray Express Quantitative software was used to quantify the spot-fluorescence intensity from the scanned images.

Protein Control Experiments

Antibodies (49D2, HPA001900, F8512, and 1D6) were printed as described above along with orientation spots and slide assay controls GFP and PBS. After printing and blocking, multiple chips were incubated overnight with 45 and 450 ng/mL of purified fibrinopeptide A (Product No. F3254; Sigma-Aldrich), 200 and 2000 ng/mL of fibrin (Product No. F5386; Sigma-Aldrich), 0.20, 200, and 2000 ng/mL of fibrinogen (Product No. F3879; Sigma-Aldrich), and serum and plasma from the same individual (Golden West Biologicals, Inc., Temecula, CA), which were diluted 1:1000, 1:10 000, and 1:100 000. Purified proteins, serum, and plasma were all diluted in 0.1% casein/PBS/GFP. The 0.1% casein/PBS/GFP was used as a blank control on additional chips. After incubation, the slides were washed and incubated at room temperature with 0.3 ng/mL of biotin-labeled antibodies 49D2, HPA001900, F5813, or 1D6. Amplification and detection of signal was done as described above.

Results and Discussion

To determine if we could distinguish between blood plasma and serum samples based on fibrinogen content, we developed a sandwich ELISA microarray assay for intact fibrinogen. Initially, control experiments were performed to evaluate antibody activity towards fibrinopeptide A, fibrin, and fibrinogen. Detection antibody 1D6 did not recognize fibrinopeptide A on microarray chips containing capture antibodies 49D2, HPA001900, F8512, or 1D6 (data not shown). Similarly, microarray chips incubated with fibrin or serum samples did not generate a signal with 1D6 as the detection antibody and 49D2, HPA001900, F8512, or 1D6 as capture antibodies. In contrast, microarray chips incubated with fibrinogen or plasma yielded a strong signal when antibodies 49D2 and 1D6 were used in the sandwich ELISA, but not when 1D6 was used as both capture and detection antibody (Figure 1). When F8512 was used as the capture antibody, the signal patterns were similar to 49D2 but the signal strength was weaker (data not shown). Surprisingly, little to no signal was observed when HPA001900 was used as the capture antibody in conjunction with 1D6 as detection antibody.

Subsequent experiments were conducted to evaluate the performance of 49D2, HPA001900, and F8512 in sandwich ELISA assays with 1D6 for distinguishing plasma from serum. Based on preliminary studies, we determined that triplicate ELISA microarray analyses would utilize less than 0.5 μ L of the original samples prior to a 500-fold dilution (data not shown), demonstrating the sensitivity of the assay and the high abundance of fibrinogen in blood [14]. As can be seen, 49D2 and F8512 capture antibodies yielded positive signals for fibrinogen in plasma when using 1D6 antibody as the detection reagent (Figure 1, Panel 1), resulting in a similar intensity between plasma and serum. However, 49D2 appeared to perform the best of all the capture reagents we tested. As mentioned above, antibody 49D2 gave a stronger positive signal, suggesting that it is the more sensitive capture reagent compared with F8512. Furthermore, for 49D2, there was no overlap in signal intensity between the serum and plasma samples, and the group means were highly significantly different ($p=1.1 \times 10^{-17}$, as determined by an unpaired, 2-tailed t-test). This is not unexpected since 49D2 is a monoclonal antibody against the fibrinopeptide A component of fibrinogen, which is proteolytically cleaved from fibrinogen by thrombin during blood clotting and serum formation [15]. As discussed above, antibody HPA001900 was unable to detect fibrinogen (except for very low signal for the serum sample in Figure 1, Panel 3) during the evaluation of this sandwich ELISA assay. F8512 consistently displayed relatively high background signal in the three serum samples shown, which brings in to question the utility of this particular antibody for distinguishing plasma and serum samples. The F8512 polyclonal antibody illustrates the problem with currently available ELISA kits. Manufacturers cannot guarantee that polyclonal antibodies used in these kits will not also detect the normal

fibrinogen break-down products created during serum formation. 1D6 was also printed as a capture antibody. As expected, the detection antibody could not bind when 1D6 was also used as the capture antibody since its antigenic site is the same.

Based on the performance of 49D2 and F8512 capture antibodies, we conducted a blind evaluation of their utility in distinguishing plasma samples from serum samples after 500-fold dilution. The identities of 10 fasting plasma, 10 non-fasting plasma, 10 fasting serum, and 10 non-fasting serum samples from the same individuals were correctly assigned with 100% specificity and sensitivity as either plasma or serum (Figure 2). The data indicate that 49D2, and to a lesser extent F8512, can successfully distinguish between unknown plasma and serum samples when using 1D6 as the detection antibody. We also analyzed plasma samples that were diluted 100-fold and 1000-fold (data not shown). These analyses show a drop in signal from the 100-fold to the 1000-fold dilution, indicating the capture antibody is not fully saturated over this range of sample dilutions. Although the 1000-fold dilution was diluted twice as much as the serum samples, this plasma sample still has a markedly higher signal than any of the 20 serum samples, consistent with the expectation that fibrinogen levels in plasma are much higher than those in serum. In addition, analyses of fasting and non-fasting plasma or serum samples from the same individuals did not indicate any affect of fasting on the fibrinogen concentrations (data not shown).

We used casein as the blocking reagent in our assay system, and this reagent appeared to work very well. Even so, portions of the casein protein have been reported to have some homology to fibrinogen [16]. Thus, it is conceivable that there could be an interaction between the anti-fibrinogen antibodies and the casein blocking protein. We did not observe any evidence that the anti-fibrinogen monoclonal antibodies used in this study had any cross-reactivity with casein, since cross-reactivity with the detection antibody would be expected to produce very high background, while cross-reactivity with the capture antibody would be expected to inhibit its activity and thereby suppress fibrinogen-specific signals. Thus, since we observe exceptionally low background signal under assay conditions that produce a strong fibrinogen signal (Fig. 1), the two antibodies used in our assay system apparently do not have any detectable reactivity with casein.

In summary, we have developed a rapid sandwich ELISA in microarray format for fibrinogen that can distinguish plasma from serum samples. Advantages of this method include the ability to use very small sample volumes (<1 μ L total for triplicate analyses), high specificity, and high throughput potential.

Acknowledgments

The authors would like to thank Dr. Roger L. Lundblad of Lundblad Biotechnology for very helpful discussions of fibrinogen assays. This work was supported in part by NIH grant DK070146 to R.D.S. and T.O.M and NIBIB grant EB006177 to R.C.Z. A portion of the work was performed in the EMSL, the Environmental Molecular Sciences Laboratory, a national scientific user facility located at Pacific Northwest National Laboratory (PNNL) and sponsored by the U.S. Department of Energy (DOE) Office of Biological and Environmental Research. PNNL is operated by Battelle for the DOE under Contract No. DE-AC06-76RLO-1830.

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Figure 1. Sandwich ELISA microarray chip design

The top-left panel shows an image of a chip incubated with a plasma sample. The next 3 images show chips that were incubated with serum samples. The table shows the positioning of the capture antibodies and other reagents that were printed on the chip. The detection antibody used in all cases was 1D6. Key: PBS, buffer saline; GFP, green fluorescent protein; A633, Alexa633-tagged immunoglobulin G.

Figure 2. Results of sandwich ELISA microarray analysis of blinded plasma and serum samples Plasma and serum samples, after a 500-fold dilution, were analyzed for fibrinogen levels using the ELISA microarray platform. The box plot of each set of sample values shows the data range from the 25th to the 75th percentile, while the horizontal line in the box represents the median value. Crossbars extend to the 10th and 90th percentile values, with outliers beyond this range shown as individual values. The p value of 10^{-17} was derived using an unpaired Student's t-test.