MEASURING BIOMARKERS FROM DRIED BLOOD SPOTS UTILIZING BEAD-BASED MULTIPLEX TECHNOLOGY

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Dried blood spots is an alternative method to collect blood samples from research subjects. However, little is known about how hemoglobin and hematocrit affect bead-based multiplex assay performance. The purpose of this study was to determine how bead-based multiplex assays perform when analyzing dried blood spot samples. A series of four experiments outline the study each with a specific purpose. A total of 167 subject samples were collected and 92 different biomarkers were measured. Median fluorescence intensity results show a positive correlation between filtered and non-filtered samples. Utilizing a smaller quantity of sample results in a positive correlation to a larger sample. Removal of hemoglobin from the dried blood spot sample does not increase detection or concentration of biomarkers. Of the 92 different biomarkers measured 56 were detectable in 100-75% of the attempted samples. We conclude that blood biomarkers can be detected using bead-based multiplex assays. In addition, it is possible to utilize a smaller quantity of sample while avoiding the use of the entire sample, and maintaining a correlation to the total sample. While our method of hemoglobin was efficient it also removed the biomarkers we wished to analyze. Thus, an alternative method is necessary to determine if removing hemoglobin increases concentration of biomarkers. More research is necessary to determine if the biomarkers measured in this study can be measured over time or within an experimental model.

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CHAPTER 1

BACKGROUND

In the 1960s a new method of blood collection consisting of filter paper cards or dried blood spots (DBS) allowed for the successful development of an assay to determine the concentration of phenylalanine within new born humans [1]. This new method of blood collection brings with it numerous advantages. DBS cards are lightweight but are not fragile, require little packing protection, can be shipped without need of refrigeration, are more cost effective, and provide less biohazard risk, as the blood is no longer in a liquid state. The use of DBS has increased to include detection of human immunodeficiency virus infection, Epstein Barr virus, and numerous interleukins [2-5].

However, despite these advantages two limitations exists that could reduce the validity of DBS biomarker quantification. First, hematocrit effects on DBS spot homogeneity as the spread of blood is affected by the percentage of hematocrit. A 50μL blood sample with hematocrit >55% will not spread across the area of the 12mm diameter boundary (standard size for DBS collection), but a sample with hematocrit <45% will spread beyond the 12mm diameter boundary [6]. If the spread of blood is affected by hematocrit then the spread of biomarkers on DBS is likely also affected. Second, the presence of hemoglobin might have negative effects on assay performance [7]. The manufactures of enzyme linked immunosorbent assays (ELISA), and bead-based multiplex assays recommended avoiding the use of hemolyzed samples.

In order to analyze DBS samples one of two methods is generally used: ELISA or liquid chromatography/mass spectrometry (LC/MS). Although both methods are reliable,

limitations exists for both. Quantifying two or more biomarkers with ELISAs requires acquiring two or more separate assay kits, increasing the necessary sample volume and cost. LC/MS has the ability to measure two biomarkers without doubling supplies and cost, but LC/MS requires an extensive budget to purchase the instrument, supplies, and maintenance purposes. Bead-based multiplex assays are a relatively new process that can be utilized to quantify a large quantity of biomarkers from plasma samples [8, 9]. However, there is limited data analyzing DBS samples with bead-based multiplex assays. Whether or not the presence of hemoglobin and hematocrit play a role in bead-based assays has yet to be determined.

The ultimate goal of our research group is to develop a method to monitor research subjects that cannot be present in the laboratory for blood sample collection. However, due to the implications from hematocrit and hemoglobin in DBS samples we are unaware how the change from ELISA or MS/LC to bead-based multiplex assays affects quantification Thus, the purpose of this study was to determine how bead-based multiplex assays perform when analyzing DBS samples.

CHAPTER 2

METHODS

Overview

A series of four experiments outline the purpose of the study each with different aims. Experiment 1 was to determine if biomarkers could be eluted and quantified using bead-based multiplex assays from filter paper. Experiment 2 determined if using four 6mm disks compared to four 12mm spots results in equal concentration values. Experiment 3 determined if the removal of hemoglobin increases biomarker median fluorescence intensity and concentration. Experiment 4 determined which biomarkers could be detected using bead-based multiplex assays.

Sample Source

The University of North Texas Institutional Review Board approved all human subject procedures, and subjects gave written and verbal consent prior to sample collection. Both men and women completed a brief medical history questionnaire to screen for any known physician diagnosed diseases. After being approved for the study, subjects reported to the laboratory in the morning (0500-0900) in a fasted state (>8-h) and abstention from exercise (>12-h) for collection of blood samples. Subject hematocrits were measured at the time of DBS collection to screen for any unknown anemic or polycythemia related blood diseases. Experiment 1 used no subjects, Experiment 2 used 12 subjects, Experiment 3 used 8 subjects, and Experiment 4 used 147 subjects.

Dried Blood Spots

Blood was collected on 903 Whatman Filter Paper cards (Whatman; Florham Park, NJ) filling the four 12mm circles spots with blood. The collection procedure followed the recommendations set forth by the European Bioanalysis Forum [10]. Following the collection, DBS cards were dried for a minimum of 2-h. Then, the cards were transferred to reseable light-protected foil bags (S-18134, ULINE; Pleasant Prairie, WI), with a 10g desiccant and a humidity indictor card (S-1547, ULINE). Sealed bags were stored at -80°C until processing. Approximately, 24-h prior to processing, sealed bags were transferred to a 2-8°C refrigerator to slowly thaw samples. The 12mm DBS were punched with a paper punch in a linear, perimeter-to-perimeter pattern producing two 6mm dried blood disks. This punching method was utilized in all four experiments.

Elution/Filtration Method

An elution buffer composed of Dulbecco's phosphate buffered saline (D5652; Sigma Life Science; St-Louis, MO), 0.1% Tween-20 (P2287; Sigma Life Science), and 0.5M NaCl (Macron Chemicals; Center Valley, PA) was prepared to elute blood contents from punched DBS. Punched 6mm samples and elution buffer, 200µL per 12mm circle spot, were transferred to a 0.45µm pore-size, 96-well polypropylene plate (MAHVN4550; Millipore, Billerica, MA) and stored at 2-8°C for 24-h. The 0.45µm pore-size plate can be stacked on top of a 96-well 10kDa membrane filter plate (MAUF1010; Millipore), and a clear 96-well round bottom plate (3371; Corning, Massachusetts, USA). The stacked plates were centrifuged (RT7 Plus, RTH-750, Sorvall; Waltham, MA) at 2000xg for 1-h. The targeted biomarkers were filtered through the 0.45µm pore plate to remove the filter paper, and then through the 10kDa membrane plate to remove the

elution buffer. The contents of the elution buffer were captured in the Corning plate. This elution and filtration method occurs in all four experiments.

Bead-Based Multiplex Assays

All bead-based MILLIPLEX® Map Kits (Millipore) were preformed according to instructions provided by the manufacturer. Briefly highlighting the key steps, seven standards were prepared in serial dilution and transferred to 96-well microtitered polypropylene plate in duplicate along with quality controls (high and low), and samples. Magnetic beads designed to adhere to specific biomarkers were added to all wells on the first day and incubated for 16-18 h on agitation in the dark and at 2-8ºC. The following day supernant were decanted, and detection antibodies labeled with biotin, and a fluorescence dye conjugated to streptavidin were added to all wells. The concentration of biomarkers were determined by the intensity of the fluorescence and results were captured with a charge-coupled device camera on the Luminex® XMAP® (MAGPIX; Austin, TX) instrument and analyzed on Milliplex Analyst (v. 5.1, Millipore). For experiments 1-3, the multiplex kit (HCVD3MAG-67k; Millipore) measured 11 biomarkers: pf4/cxcl4, α1-acid glycoprotein, serum amyloid p-component, c - reactive protein, adipsin, fetuin a, haptoglobin, l-selectin, fibrinogen, α2-macroglobulin, and von willebrand factor. In Experiment 4 we utilized an additional 6 multiplex kits for a total of 92 biomarkers.

Experiment 1

The purpose of experiment 1 was to determine if biomarkers could be eluted and quantified from filter paper using bead-based multiplex assays. The sample source consisted of the standard provided from the multiplex assay used to prepare the

concentration curve. The filter paper consisted of the same filter paper used to collect blood. First, the standard was reconstituted with deionized water followed by a serial dilution that produced an additional 6 standards. The concentration curve was determined with non-filtered standards by the instructions provided in the bead-based multiplex assay manual. Thereafter, 50μL of the non-filtered standards were spotted onto seven different 12mm circles of filter paper. Spotted standards were stored at ambient room temperature to dry for 8-h. Two 6mm disks were punched from each 12mm circle filter paper and transferred with elution buffer to the 0.45μm pore-size plate. The remaining centrifugation and filtration steps occurred as described in the above Elution/Filtration Method. Then, 50μL of the now filtered standards were transferred to the same bead-based multiplex assay used to establish the concentration curve. Pearson bivariate correlation test was used to determine correlation between non-filtered standard concentrations and filtered standard concentrations. Significance was set to $p<0.05$.

Experiment 2

Using the entire 12mm spot appears to be a method to avoid the different concentration of biomarkers from the center to the perimeter of the spot [11]. However, using the entire spot leaves no sample for reanalysis. Using a smaller quantity of DBS leaves room for reanalysis, but it is unknown if using less sample effects the signal-noise ratio. The purpose of this experiment was to determine if using four 6mm disks compared to four 12mm spots results in similar biomarker concentration. Twelve human subjects each donated a total of six 12mm spots of blood onto DBS cards. Each sample was split into two different quantities one consisting of four 12 mm spots and the

following consisting of two 12mm spots. The first four 12mm spots cut in their entirety from the DBS card. The last two 12 mm spots were punched to yield 4, 6mm discs. The elution buffer volume was standardized to 200 μL per 12 mm circle, which should result in different volumes of a similar concentration. Both quantities were eluted in a 0.45μm pore-size plate. The remaining centrifugation and filtration steps occurred as described in the above Elution/Filtration Method section. Elutions from the four 12mm spots and four 6mm disk were analyzed with the same bead-based multiplex assay. The median florescent intensity and concentration of each biomarker from the four 6mm disk group and the four 12mm spot group were statistically analyzed with paired *t*-test and Pearson bivariate correlation. Significance was set to $p<0.05$.

Experiment 3

The third experiment determined if the removal of hemoglobin increased biomarker median fluorescence intensity and concentration. Eight human subjects provided four 12mm spots of blood, which were divided equally to compare the impact of hemoglobin removal. Subject samples were sub-divided based on elution technique: 1) basic method (with hemoglobin) and 2) basic method with additional steps to remove hemoglobin (without hemoglobin). The with hemoglobin samples were prepared as described as in the Elution/Filtration Method section. The without hemoglobin samples were punched producing four 6mm disk and transferred with elution buffer to the 0.45μm pore-size plate. The 0.45μm pore-size plate was stacked above the round-bottom plate for centrifugation (2000xg for 1-h) to remove filter paper. Elutions were then transferred to a 50kDa filtration tube (UFC505096, Millipore) and centrifuged (14000xg for 1-h). The filtrate (located below the membrane) was transferred to a

10kDa membrane plate stacked above a round-bottom plate and centrifuged (2000xg for 1-h). After the final spin, the concentrated proteins located above the membrane were reconstituted with 90 μL of nuclease-free water. In order to assess the effectiveness of hemoglobin removal, the elutions were tested prior to and after passage through the 50kDa membrane using an enzymatic assay (H7504, Pointe Scientific; Canton, MI) on an automated chemistry analyzer (Chemwell-T; Awareness Technologies INC; Palm City, FL). Both the sample preparations (i.e. with and without hemoglobin) were analyzed with the same bead-based multiplex assay described in Experiments 1 and 2. The median fluorescent intensity and concentration of each biomarker were statistically compared with paired *t*-test and Pearson bivariate correlation. Significance was set to $p<0.05$.

Experiment 4

The purpose of this final experiment was to identify which DBS biomarkers could be detected using bead-based multiplex assays. A total of 147 unique subject samples were used to complete experiment 4. All elutions were completed as described previously using 2, 12mm spots. Eluted DBS samples were analyzed in duplicate using several different bead-based multiplex assay kits: Human Metabolic Hormone (HMHMAG-34K), Human CVD Panel 2 (HCVD2MAG-67K), Human CVD Panel 3 (HCVD3MAG-67K), Human Neurodegenerative Panel 1 (HNDG1MAG-36K), Human Neurodegenerative Panel 3 (HNDG3MAG-36K), Human Cytokine/Chemokine (HCYTOMAG-60K), and Human High-Sensitivity T cell (HSTCMAG-28SK). These kits were selected because the containing biomarkers were relevant to ongoing research in our laboratory. Results were categorized into quartiles (100-75%, 74.9-50%, 49.9-25%,

and 24.9-0%) depending on the number of times a given biomarker was detected (i.e. the MFI was greater than the lowest standard). For example, when a biomarker was measured and detected in 8 of 10 samples, this would be classified as 80% detectable (1st quartile).

CHAPTER 3

RESULTS

Experiment 1

Experiment 1 aimed to determine if biomarkers could be recovered from DBS cards for measurement with bead-based multiplex assays. We expected to find a strong positive correlation and >50% recovery across all biomarkers. The correlation results using median fluorescence intensity returned positive R-values that were significance among all biomarkers (Fig 1, Tbl 1). The highest ranging from α2-macroglobulin (R = 0.999) and the lowest value Adipsin (R = 0.985; Fig. 1). A similar, significant positive R-value was found when comparing biomarkers as a function of calculated concentration across all biomarkers with the exception of Adipsin (R = 0.990) and Serum Amyloid protein (R = 0.992) (Fig 2).

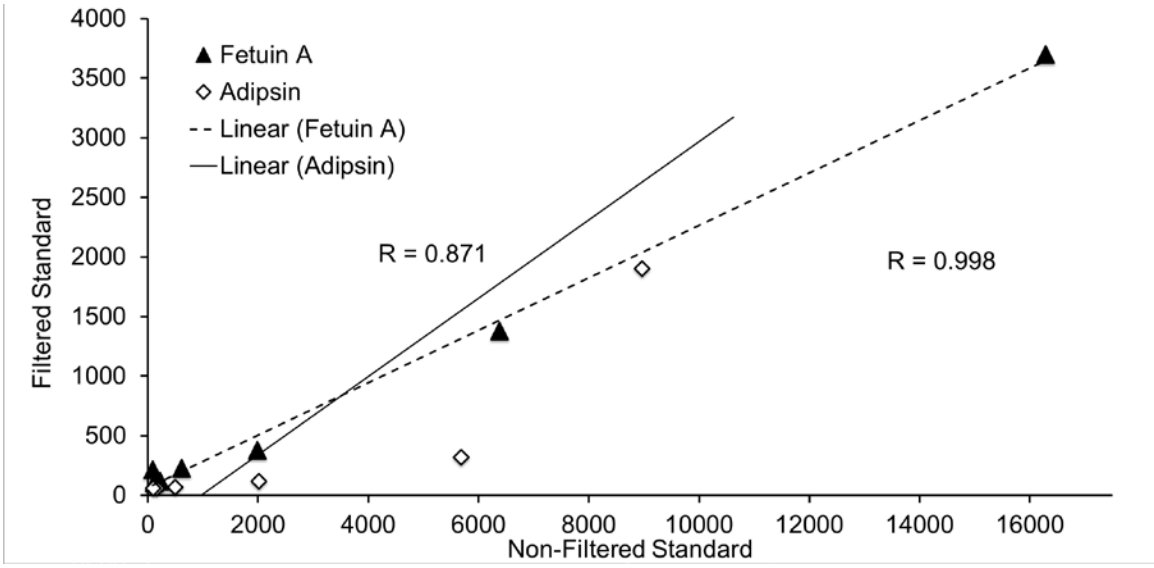

Figure 1. Highest and lowest results of correlation from median fluorescence intensity values.

Table 1

Correlation results from experiment 1 using concentration values.

Biomarker	n	R Value	Significance
Fetuin A	7	0.998	0.000
CRP	7	0.966	0.000
AGP	7	0.934	0.002
L-Selectin	7	0.937	0.002
SAP	7	0.932	0.002
α2-Macroglobulin	7	0.929	0.002
vWF	7	0.924	0.003
PF4/CXCL4	7	0.902	0.005
Fibrinogen	7	0.885	0.008
Haptoglobin	7	0.875	0.010
Adipsin	7	0.871	0.011

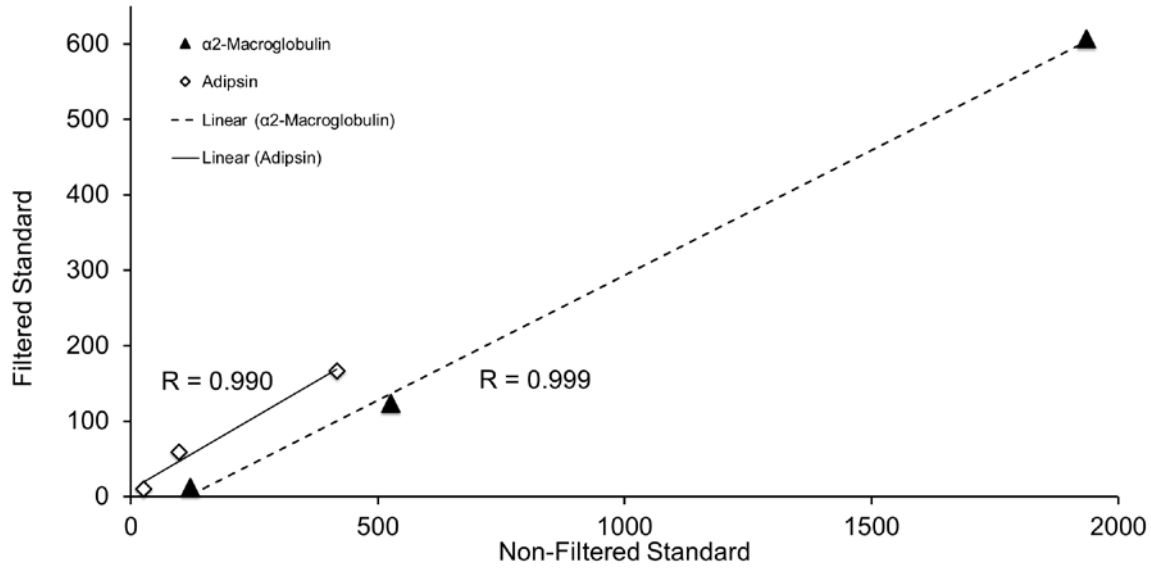

Figure 2. The highest and lowest correlation values from experiment 1 using concentration values.

Table 2

Correlation results from experiment 1 with concentration values.

Biomarker	n	R Value	Significance
PF4/CXCL4	4	.999	.001
AGP	2	.999	.000
SAP	3	.992	.082
CRP	4	.999	.000
Adipsin	3	.990	.087
Fetuin A	6	.997	.000
Haptoglobin	2	.999	.000
L-Selectin	3	.999	.028
Fibrinogen	4	.998	.036
α2-Macroglobulin	3	.999	.023
vWF	3	.998	.035

Note. All biomarkers were positively correlated with significance with the exception of serum amyloid protein and adipsin. A number of samples were excluded from the data due to low concentration and were not detectable.

Experiment 2

Experiment 2 aimed to determine if using four 6mm DBS disks compared to four 12mm DBS spots results in equal concentration values. We hypothesized that there would be a strong positive correlation and no significant differences between the two sample quantities. When comparing median fluorescent intensity of biomarkers we found a positive correlation among all biomarkers except Adipsin (R = 0.143) and α2-macroglobulin (R = 0.573; Tbl. 3). The paired *t*-test results show significant differences among a majority of biomarkers with the exception of α1-Acid Glycoprotein (*p* = 0.954), Fetuin A (*p* = 0.573), α2-macroglobulin (*p* = 0.885), and Von Willebrand Factor (*p* = 0.731; Fig. 3). When comparing concentration, we found that PF4/CXCL4, Serum Amyloid P-component, and Fetuin A were not detectable (i.e. their median fluorescent intensity was above that of the highest standard). Correlation values of biomarker concentration ranged from low (α1-Acid Glycoprotein; R = 0.558) to high (C-reactive protein; R = 0.978; Tbl. 4). The paired *t*-test showed no significant differences in a majority of the biomarkers with the exception of α1-Acid Glycoprotein (*p* = 0.003), Haptoglobin (*p* = 0.002), and α2-macroglobulin (*p* = 0.001; Fig. 4).

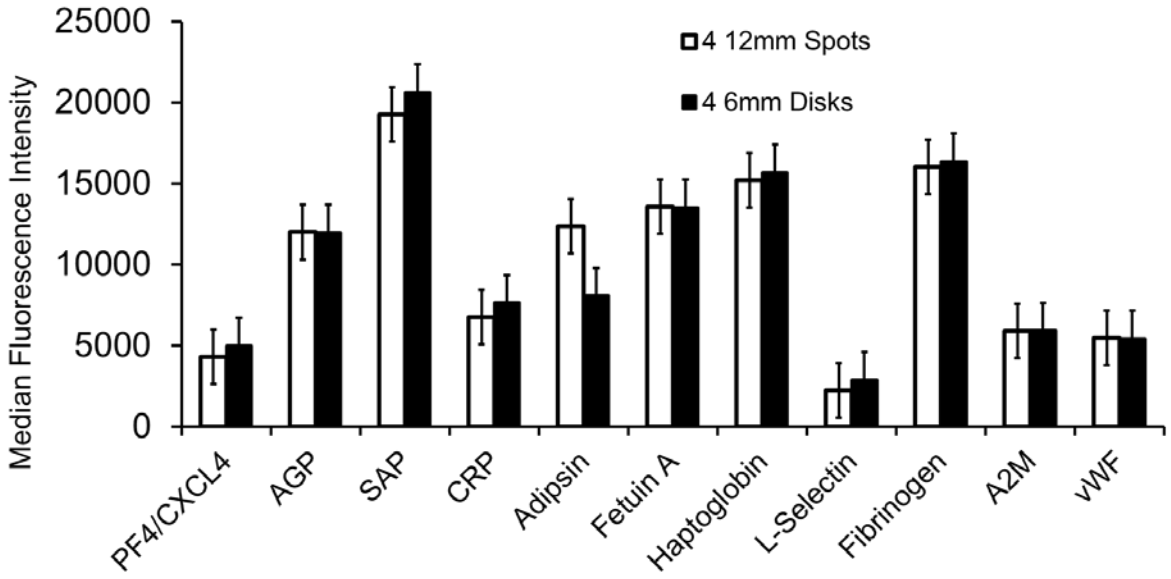

Figure 3. Experiment 2 median fluorescent intensity paired *t*-test results. Median fluorescent intensity results between the two differing quantities of DBS. With the exception of AGP, Fetuin A, α2-Macroglobulin, and vWF all remaining biomarkers were statistically different despite them being well within their standard error ranges.

Table 3

Experiment 2 median fluorescent intensity correlation and paired t-test results.

Biomarker	R Value	Significance	*t*-test Significance
PF4/CXCL4	.750	.005	<.0001
AGP	.874	.000	.954
SAP	.940	.000	<.0001
CRP	.619	.032	<.0001
Adipsin	.143	.657	.008
Fetuin A	.778	.003	.573
Haptoglobin	.896	.000	<.0001
L-Selectin	.711	.009	<.0001
Fibrinogen	.837	.001	<.0001
α2-Macroglobulin	.573	.051	.882
vWF	.976	.000	.731

Note. A majority of the biomarkers were significantly correlated with the exception of Adipsin and α2-Macroglobulin. Paired *t*-test results show significant differences, but Figure 2 shows the difference are well within their standards errors.

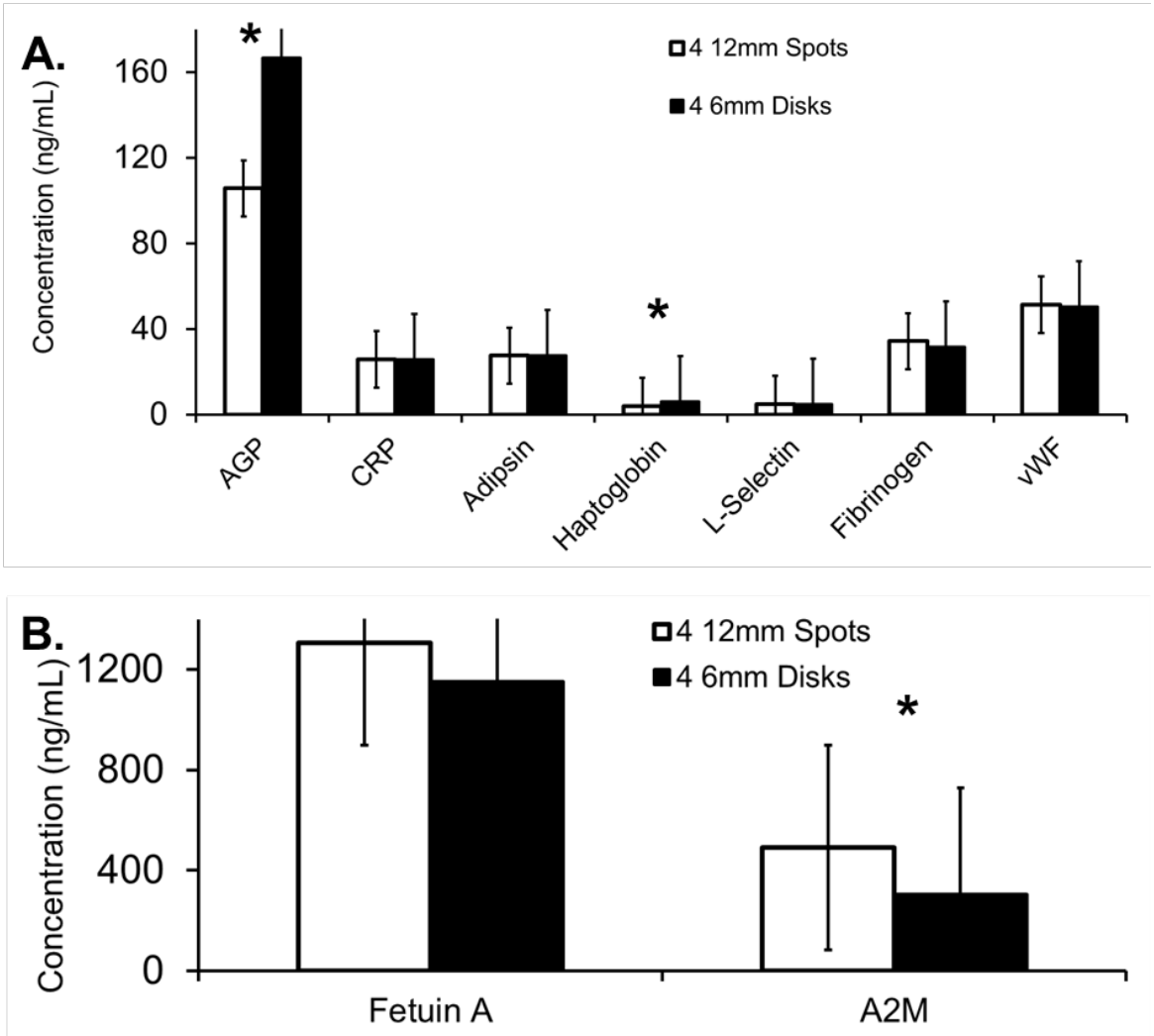

Figure 4. Experiment 2 paired *t*-test results. Concentration results between the two quantities of experiment 2. Median flourescent intensity values exceeded the concentration curve for serum amyloid protein and pf4/cxcl4, thus, they are not present on this figure. The table is split into two to visually represent the low concentration biomarkers apart from the high concentration biomarkers.* $p < 0.05$

Table 4

Experiment 2 correlation and paired t-test results with concentration values.

Biomarker	R Value	Significance	*t*-test Significance
PF4/CXCL4			
AGP	.558	.250	.003
SAP			
CRP	.978	.001	.821
Adipsin	.711	.113	.935
Fetuin A	.722	.487	.180
Haptoglobin	.705	.118	.002
L-Selectin	.917	.010	.502
Fibrinogen	.931	.069	.864
α2-Macroglobulin	.828	.042	<.0001
vWF	.976	.001	.670

Note. Due to insufficient sample sizes the significance for most biomarkers were > 0.05 despite positive correlation values. Paired *t*-test results show most biomarkers are not significantly different.

Experiment 3

For experiment 3, we hypothesized that the removal of hemoglobin would increase biomarker median fluorescence intensity and concentration. Our technique to remove hemoglobin removed 100% of intact hemoglobin (Pre-Filter: 2.14g/dL ±0.17; Post-Filter: 0.0g/dL ±0.01). When testing for correlation based on median fluorescent intensity significance was only found for von willebrand factor ($p < 0.001$; Tbl 5). No other biomarkers were significantly correlated between the two sample preparations. When examining differences with the paired *t*-test, significance was found for all biomarkers except Adipsin (R = 0.796) and α2-macroglobulin (R = 0.929; Fig. 5). When converting to concentration, significant correlations were found for AGP (R = -8.38; P=0.019) and serum amyloid protein a (R = -0.857; P = 0.014).When examining *t-test* comparisons, all biomarkers except adipsin ($p = 0.769$), fibrinogen ($p = 0.079$), α2-macroglobulin ($p = 0.874$), and Von Willebrand Factor ($p = 0.193$; Fig. 6) were different.

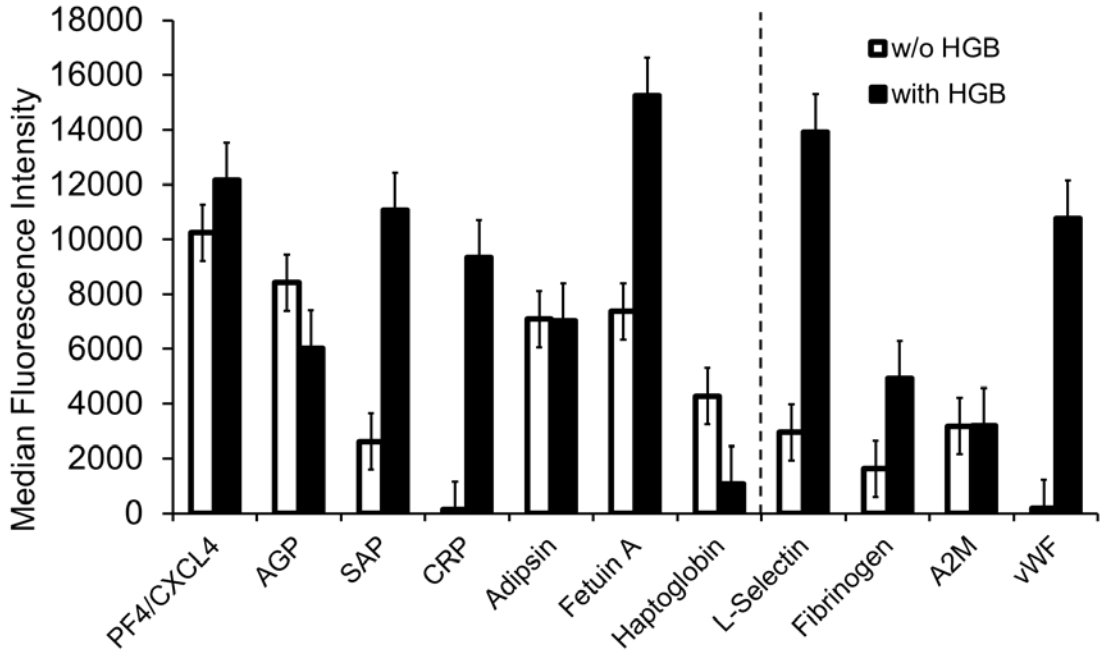

Figure 5. Median fluorescent intensity results of samples with and without hemoglobin. The biomarkers are arranged in order of smallest to largest in terms on molecular weight in kDa. The dotted line represents 50kDa, beyond this line it was expected that biomarker detection would greatly decrease as they filter from the sample along with hemoglobin. Biomarkers below 50kDa also show a negative influence from hemoglobin removal.

Table 5

Median fluorescence intensity results from experiment 3.

Biomarker	R Value	Significance	*t*-test Significance
PF4/CXCL4	.650	.350	.003
AGP	-.357	.643	.011
SAP	-.616	.384	.001
CRP	.673	.327	<.0001
Adipsin	-.259	.741	.769
Fetuin A	-.416	.584	.002
Haptoglobin	-.509	.491	<.0001
L-Selectin	.141	.859	<.0001
Fibrinogen	-.734	.266	.079
α2-Macroglobulin	-.749	.251	.874
vWF	-1.000	.000	.193

Note. No positive correlation with significance among any of the biomarkers. In addition, all biomarkers were significantly different with the exception of adipsin and α2-macroglobulin.

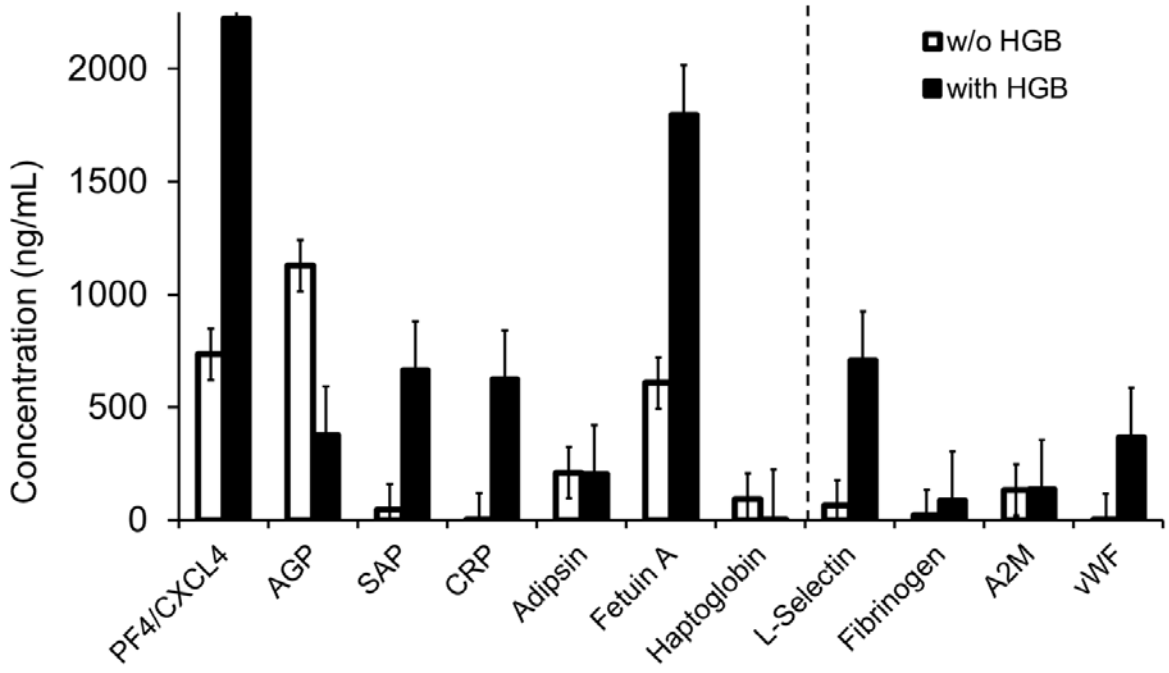

Figure 6. Concentration results from samples with hemoglobin and without hemoglobin. Similar to Figure 4, the dotted line represents the 50kDa point and biomarkers are listed in order of least to greatest. The results are similar to fluorescence intensity as the removal of hemoglobin also removed biomarkers.

Table 6

Concentration results from experiment 3. Identical results to table 4.

Biomarker	R Value	Significance	*t*-test Significance
PF4/CXCL4	.230	.585	<.0001
AGP	-.838	.019	<.0001
SAP	-.857	.014	<.0001
CRP	.609	.109	<.0001
Adipsin	-.299	.473	.796
Fetuin A	-.489	.219	<.0001
Haptoglobin	-.718	.069	<.0001
L-Selectin	-.123	.771	<.0001
Fibrinogen	-.674	.067	.014
α2-Macroglobulin	-.485	.223	.929
vWF	-.068	.872	<.0001

Note. No positive correlation with significance, and all biomarkers are significantly different.

Experiment 4

For experiment 4 we hypothesized that not all biomarkers would be detectable using bead-based multiplex assays. In order to classify frequency of detection, biomarker concentration values were calculated and biomarkers were classified into quartiles based on frequency of detection. For example if a given biomarker were detected in 8 of 10 samples, this biomarker would have a detection percentage of 80% and be classified to the 1st quartile. Using this classification scheme, we found that 56 biomarkers were detected in the 100-75% range, 6 biomarkers in the 74.9-50% range, 9 biomarkers in the 49.9-25% range, and 21 biomarkers in the 24.9%-0% range (Tbl. 7).

Table 7

The detection frequency of 92 individual biomarkers measured from DBS samples

Human Neurodegenerative Panel 1		Human Cytokine/Chemokine		Human High-Sensitivity T Cell	
Biomarker	% Detected	Biomarker	% Detected	Biomarker	% Detected
α2 - Macroglobulin	100	Fractalkine	52	Fractalkaline	96
Complement C3	100	GM-CSF	33	GM-CSF	75
Prealbumin	100	IFNγ	24	IFNγ	71
Complement Factor H	100	IL-10	0	IL-10	34
Apo-A1	80	IL-12P70	48	IL-12P70	54
Apo-E	30	IL-13	0	IL-13	25
Human Neurodegenerative Panel 3		IL-17A	0	IL-17A	32
BDNF	100	IL-1β	52	IL-1β	98
Cathespin D	100	IL-2	0	IL-2	11
sICAM-1	100	IL-4	0	IL-4	41
MPO	100	IL-5	0	IL-5	5
PDGF-AA	100	IL-6	0	IL-6	23
RANTES	100	IL-7	33	IL-7	84
NCAM	100	IL-8	100	IL-8	100
PDGF- AB/BB	100	MIP-1α	19	MIP-1α	75
sVCAM-1	100	MIP-1β	100	MIP-1β	100
PAI--1	0	TNFα	62	TNFα	98
Human Metabolic Hormone					
Leptin	96	TNFβ	29	IL-21	100
MCP-1	100	EGF	100	IL-23	54
Human CVD Panel 3		Eotaxin	100	ITAC	100
α2 - Macroglobulin	100	FGF-2	86	MIP3α	100
CRP	100	Flt-3L	0		
Fetuin A	100	G-CSF	86		
AGP	100	GRO	100		
Fibrinogen	100	IFNα2	19		
L-Selectin	100	IL-12P40	0		
SAP	100	IL-15	5		
Haptoglobin	100	IL-1RA	100		
PF4/CXCL4	100	IL-1α	100		
Adipsin	100	IL-3	0		
vWF	100	IL-9	10		
Human CVD Panel 2		IP-10	100		
sICAM-1	100	MCP-1	95		
P-SELECTIN	100	MCP-3	5		
sVCAM-1	100	MDC	100		
SAA	100	sCD40L	100		
		TGF-α	10		
		VEGF	100		

Note. If a biomarker was detected in 8 of 10 samples then its frequency of detection would be calculated as 80% and assigned to the 1st quartile.

CHAPTER 4

DISCUSSION

The primary purpose of the present study was to determine the potential to use bead-based multiplex assay to measure biomarker concentration in eluted DBS samples. In order to achieve this aim, we completed a series of experiments designed to evaluate and increase the efficiency of various aspects of DBS collection, elution, and analysis. From these efforts, we have confirmed that biomarkers can be eluted DBS cards and quantified using bead-based assays. Second, that when standardizing elution buffer according to quantity of DBS punched that a smaller quantity (2,12mm) of DBS yields similar results as a larger quantity (4, 12mm). The presence of hemoglobin in eluted DBS samples does not necessary interfere with biomarker detection in bead-based assays; however, more research is needed to confirm this is the case. Finally of the 92 biomarkers screened in the present study, we found that 56 were detectable in > 75% of the measured samples.

Consistently with previous studies that have shown that virus is eluted from DBS cards, we confirmed that biomarkers spotted onto DBS cards are successfully eluted and detected using bead-based multiplex assays [2-5, 12]. Despite consistent elution of standards and high percent recoveries, it is of note that only ~50% of the protein in the serum component of the DBS is eluted. This later effect is likely the result of the filtration process used in the present study. Care should be taken when selecting biomarkers for DBS analysis; specifically targeted biomarkers should be screened on a case-by-case basis to determine their validity in DBS samples.

De Kesel et al. (PMID: 25158957) reported that the hematocrit percentage of a patient's sample greatly affects the spread of blood on DBS cards. Such a spread can be mitigated by using the entire 12mm spot rather than commonly used punching techniques. Also, Prince et al. previously demonstrated that when elution buffer concentration is standardized that smaller quantities of DBS were associated with decreased the measurement of antibody concentration using an ELISA test [13]. Our study aimed to address previous findings by comparing entire 12mm DBS spots to 6 mm punched DBS disks. When comparing concentration, we found no statistically significant correlations for all but 1 biomarker. When comparing raw median fluorescent intensity we found strong correlations between the two quantities. Consistent with previously published data [2-5, 12], care should be taken when selecting a quantity of DBS to use for analysis. The use of modified DBS cards that have the potential to mitigate hematocrit effects is likely warranted based on the findings from our study and others.

Hemoglobin is routinely mentioned as a confounder of bead-based multiplex assay performance [7]. Based on these previous findings, we hypothesized that removal of intact hemoglobin from the eluted DBS sample would improve biomarker detection. We utilized a membrane filtered based technique to remove hemoglobin, but a negative side effect of this approach was that it also removed significant volumes of our targeted biomarkers. Further complicating our outcome was that removal of hemoglobin did not necessary improves assay performance, but in fact reduced assay performance by lowering the total protein concentration in the DBS elutes. Other investigators have speculated that hemoglobin contamination of eluted DBS is a concern; however, few

current technique allow for its removal. One technique that has been proposed and may represent a viable approach is the using of dried serum or plasma samples rather than whole blood. More research is needed to validate these alternate sample source.

Previous research using a mass spectrometer detection technique has demonstrated that a variety of proteins are present and detectable in DBS samples (REF). Based on this previous knowledge, we speculated that similar to mass spectrometry, bead-based assays would also be able to detect a large array of protein biomarkers. Consistent with this speculation, 78% of the 92 biomarkers screened had a frequency of detection greater than 50%. For the present comparison is opted to only use fasting blood samples from healthy subjects. The purpose of selecting this population was to validate that DBS biomarkers could be detected in healthy individuals where they tend to be present a low concentrations. Extension of the present findings suggests that in disease states, at the very least all the biomarkers detected in the present study would also be present albeit at higher concentrations. Another interesting, but not unexpected finding was that multiplex kits that were designed to detect serum samples worked better for DBS than kits designed for cell culture supernants. In the present study this can be observed by comparing the cytokine/chemokine and high-sensitivity T cell kits. For these kits, we found that similar biomarker profiles, but much greater detection frequencies with the high-sensitivity kit. These findings highlight that care should be taken when selecting bead-based kits to ensure that they are at least compatible with the low biomarker concentrations that are anticipated from DBS samples. Also, more research is necessary to determine biomarkers in the lowest

detection quartile become detectable in clinical patients or following experimental treatment.

In summary, the present study demonstrates that bead-based multiplex assays may be a useful tool for measuring biomarkers from DBS samples. Consistent with previously reported findings, care should be taken to measure and control for patient sample hematocrit and hemoglobin concentration. Both of which are known to influence different aspects of assay performance. Finally, we demonstrated that of our 92 biomarkers measured, 78% are detectable in at 50% of the blood samples screened. Since our study included fasted, healthy individuals, more research is needed to apply the bead-based analysis described in this report to clinical populations undergoing experimental treatment.

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