Diagnostic Challenges: Advancing Assay Design

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Statement by author

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5/17/2018
Date

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PROLOGUE

A unique aspect of the Professional Science Masters Applied Bioscience program is to immerse future graduates into the fields of scientific research inclusive of both business and basic science. At the culmination of the program, an 8 credit hour internship requirement proved to be an invaluable opportunity for me to experience a broad range of scientific inquiry. Throughout the duration of the internship, I was able to learn and master laboratory techniques, adhere to federal and state regulations, practice communicating my results, and ultimately be a contributing member of a collaborative team. In return, I aided my supervisors by completing projects and assigned tasks that was of great value and/or interest to the company. Internships can be done at one company or split to be completed at two separate industries. I was fortunate enough to be able to experience two companies, one focusing more on research and assay development (MSDx) and the other focusing on diagnostic tools to add to their testing services (Pharos Dx).

The first internship was with MSDx, Inc., a research company aiming to provide diagnostic solutions for neurodegenerative disorders. The researchers were interested in moving a first generation assay to a second generation assay for greater specificity in detecting tau, a protein which is primarily located within the brain and a proven marker for brain degeneration [10, 11]. Their first generation assay, an indirect ELISA, is compatible with their current anti-tau monoclonal and polyclonal antibodies. My responsibilities were to search for additional anti-tau antibodies that could be used as pairs in a sandwich assay and would detect an epitope within the tau amino acid sequence of interest (c-terminus of the protein). At the conclusion of the internship, I created a table of commercially available antibodies and provided it to the company (table 1 within the paper). I was also able to demonstrate how the first generation assay worked to model the first steps that would be taken when testing a new antibody for assay development (figure 4).

The second internship took place at Pharos Dx., which is a diagnostic company utilizing liquid-chromatography mass-spectrometry as part of their testing services. Pharos Dx was looking to offer microsampling devices to their clientele as it would help veterinarians to easily collect samples from animals. Microsampling has a disadvantage with the hematocrit where an unknown volume of blood would affect the overall spreading and homogeneity of the sample across the filter paper [43, 46]. My responsibility during this internship was to research the various microsampling tools currently out on the market. With the amount of published papers available, my objective was to sort through the validated and completed experiments to make a recommendation to Pharos Dx regarding the microsampling device to pursue and explore further. At the conclusion of the internship, I summarized my findings (presented within this paper) and suggested Pharos Dx begin confirmatory testing of using a potassium based algorithm to remove the hematocrit bias associated with dried blood spot cards [52, 64].

In conclusion, each internship was a full experience of new content and laboratory techniques. I gained a greater appreciation for what scientists have to go through to create commercial products to be used in disease detection and monitor overall health. With MSDx, Inc., I was able to learn a substantial amount about brain disorders and the recent findings (2015) of lymphatic vessels in the brain [5, 6]. With Pharos Dx., even though the focus was researching microsampling for a diagnostic tool, I learned more about the regulatory side of research and how to trust published results based on the validation of the procedures that were run. It was my

goal that I would be able to contribute tangible products for these companies, as both companies truly helped me grow as a scientist.

Chapter 1: MSDx Inc's Window into the Brain™ Tau protein assay- Moving from first generation assay to a second generation assay

ABSTRACT:

MSDx Inc., a personalized medicine company, develops blood testing products for brain diseases. The detection by MSDx of brain debris laden phagocytes re-entering the blood circulation after brain injuries was strengthened with confirmation of lymphatic vessels discovered in the brain of mice in 2015. Debris in phagocytes contains significant information (in the form of brain specific proteins and their fragments) about disease activity after injury has occurred. Deciphering the information contained within the circulating phagocytes has made MSDx dedicated to providing neurologists with critical data that is useful in diagnosis, monitoring, and assessing therapeutics response. With this goal in mind, MSDx's first patented blood test panel technology known as **WINDOW INTO THE BRAIN™** which offers a real-time view of pathological processes and disease activity in the brain. Currently the assay utilizes an indirect ELISA detecting the protein of interest known as tau which has been associated with neuro degeneration. The focus of this project was to provide preliminary data to advance the assay platform onto a sandwich ELISA. To that end, computer based searches of commercially available antibodies with epitopes within the tau amino acid sequence of interest were conducted. Preliminary indirect assays were also completed using MSDx's current polyclonal and monoclonal antibodies. The findings reveal a number of potential antibody pair candidates, which would complement the antiserums currently in use and potentially replace them.

INTRODUCTION:

MSDx is a personalized medicine company developing blood testing products for brain diseases including multiple sclerosis (MS), Parkinson's disease (PD), and traumatic brain injuries (TBI). The company's first patent was issued for its WINDOW INTO THE BRAIN™ (WIB) blood panel technology which offers a less invasive method of detecting neurological conditions through analysis of circulating phagocytes [1]. The technology is sometimes referred to as a "non-invasive brain biopsy". MSDx assays can monitor demyelination, neurodegeneration and leukocyte transmigration biomarkers [1]. Since millions of people are affected by brain disorders, MSDx's goals are to provide a blood test panel (WIB) that has biomarkers for disease detection before excessive damage takes place.

When injury to the body occurs, phagocytes, predominantly macrophages are recruited to a site of infection or injury through the release of chemokines (cell signaling system) [2, 3, 4]. When macrophages reach their intended targets, the damaged cellular material is then engulfed through phagocytosis [2, 3, 4]. The debris, which is the breakdown of damaged cells, provides information regarding the damage. MSDx in 2007 discovered phagocytes that had previously entered the brain and engulfed debris re-entered the peripheral circulation [1, 22]. When presented with this information, the general response received from the US patent examiner and others to the MSDx team was "this is not possible because there is no lymphatic system in the brain" (quoted by Dr. Ram Nayak, inventor). MSDx's discovery contradicted decades of

immunological dogma and the discovery in 2007 could have been viewed as the smoking gun indicating that lymphatic vessels do indeed exist in the brain. Finally, in 2015, lymphatic vessels were discovered in the brain, which validated MSDx's findings of brain antigen loaded macrophages in the peripheral blood and how they got there [5, 6].

When injury to the brain occurs, such as a concussion, the immune system recruits cells to the damaged sites [4]. When macrophages engulf the damaged matter, they re-enter the circulation, can be drawn out in the peripheral blood, and can potentially provide pertinent information regarding brain pathology [5, 6]. Once the macrophage engulfs the damaged material, its' job is to destroy the debris and recycle the components [2, 3, 4]. Consequently, if debris components are detected in the peripheral blood macrophage, it is an indication of an active degenerative process as debris present in the macrophage is only detectable for a short period of time (days to couple of weeks) before it is completely digested [1 5, 6].

One protein of great importance in the normal function of nerve cells is the tau protein [7]. The tau protein is widely known in Alzheimer's disease as it forms neurofibrillary tangles when it is phosphorylated [7, 8]. The normal function of the tau protein is to stabilize axonal microtubules in neurons [8, 9, 10, 11]. High levels of tau protein is linked to poor recovery after head trauma and this knowledge, along with MSDx blood test panel, could reveal biomarker expression patterns to predict who will have a concussion that improves and who will develop late sequelae from the injury.

The focus of the internship project at MSDx was to aid in the process of moving the first generation tau assay into a second generation assay that could be licensed onto commercial instrumentation. The first generation assay is an indirect ELISA (enzyme-linked immunosorbent assay) where purified patient material (lysates) are coated onto an assay surface (96-well plate) and then a primary antibody that reacts with a particular debris component such as tau is incubated with the immobilized patient sample (antigen source). After washing off unbound material, a secondary antibody which is bound to an enzyme tag (chromogenic) is added. The enzyme incubated with an appropriate chemical substrate creates a color and the intensity is related to the amount of specific protein present, this method is demonstrated further into this report. The present goal for MSDx is to create a second generation assay in the sandwich ELISA format because most commercial immunoassay platforms use sandwich technologies (higher sensitivity and robustness) [19, 20]. An indirect ELISA has the antigen of interest indirectly detected through the use of an enzyme tagged secondary antibody. Whereas a sandwich ELISA captures the antigen of interest between two primary antibodies that are specific for a non-overlapping epitope of the antigen. An example of an indirect assay as opposed to a sandwich assay can be seen in figure 1 below [19, 20].

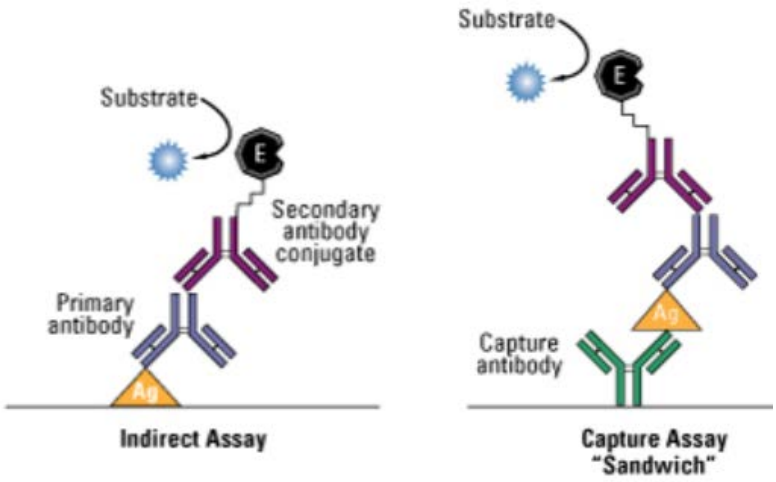

Figure 1. Indirect Assay vs a Sandwich Assay [19, 20] retrieved from Thermo Fisher Scientific.

To achieve this goal of moving first generation assay to a secondary sandwich assay requires availability of antibody pairs that can recognize the antigen of interest. Consequently, the majority of this internship was spent searching for commercially available antibodies that react with tau and specifically for antibodies where the epitope is known (MSDx project A). This report will also demonstrate the methods used currently upon receipt of a sample (cellular processing tube with whole blood) to provide detection of the tau protein with an indirect ELISA (MSDx project B).

MSDx Project A - Literature Search Methods:

To begin searching for antibodies that react with tau, a computer based literature search was conducted. Keywords used, "Tau antibodies" and "Tau monoclonal antibodies" were searched in google for publications and commercial information for antibodies on a variety of scientific sites such as Thermo Fisher, ALZFORM, BioRad, and Science Direct. Once a polyclonal/monoclonal antibody was found, further information was needed to determine if the epitope was known. A monoclonal antibody is sought out as it binds to a single epitope on an antigen rather than multiple epitopes spanning over a larger area like polyclonal antibodies (although polyclonal antibodies were not ruled out). This can be demonstrated in figure 2 below [21]:

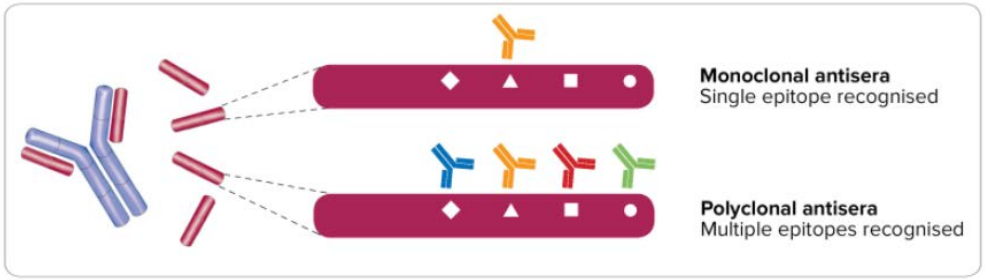

Figure 2. Monoclonal vs polyclonal antibodies and their epitopes [21].

The mature tau amino acid sequence, as well as the epitope of interest can be seen in figure 3 below [12]. The mature tau is 441 amino acids long with the epitope of interest (199 amino acids) located near the C-terminus end of the protein which is bolded and highlighted in yellow. Commercially available antibodies were primarily documented if they were found to react anywhere within the area of interest.

MAEPRQEFEVMEDHAGTYGLGDRKDQGGYTMHQDQEGDTDAGLKESPLQTPTEDGSE
EPGSETSDAKSTPTAEDVTAPLVDEGAPGKQAAAQPHTEIPEGTTAEEAGIGDTPSLEDE
AAGHVTQARMVSKSKDGTGSDDKKAKGADGKTKIATPRGAAPPGQKGQANATRIPAK
TPPAPKTPPSSGEPPKSGDRSGYSSPGSPGTPGSRSRTPSLPTPPTREPKKVAVVRTPPKSP
SSAKSR**LQTAPVPMPDLKNVKSKIGSTENLKHQPGGGKVQIINKKLDLSNVQSKCG**
SKDNIKHVPGGGSVQIVYKPVDLSKVTSKCGSLGNIHHKPGGGQVEVKSEKLDFKD
RVQSKIGSLDNITHVPGGGNKKIETHKLTFRENAKAKTDHGAEIVYKSPVVSGDTSP
RHLSNVSSTGSIDMVDSPQLATLADEVSASLAKQGL

Figure 3: Tau-441 Full Mature Protein Amino Acid Sequence [12]
(Region of interest: **amino acids 243-441**)

The rabbit polyclonal antibody that MSDx currently uses (will be described as PC1for proprietary purposes) in the first generation assay was generated by immunizing with the epitope of interest of tau shown in figure 3 above (highlighted yellow). Screening antibodies against epitopes present in this sequence is one strategy that could yield monoclonal antibodies for the second generation assay. Another strategy would be to purchase the commercially available antibodies (dependent on funding) and screen them for reactivity with lysates of pathological peripheral blood mononuclear cells (PBMCs).

Project A - Literature Search Results:

Based on the literature search, figure 4 below identifies antibodies found with known epitopes. In many cases, different antibodies exist that have overlapping epitopes found within immunizing sequences for other antibodies. This is reflected by the color coding, underlining and bolded sequences seen in figure 4 and table 1. Table 1 demonstrates where the antibodies are located within the sequence of interest including overlapping epitopes found to react with different antibodies (distinguished by color coding, underlining, and bolding of appropriate amino acid sequence). Project B demonstrates how an indirect ELISA is used to screen polyclonal and monoclonal antibodies for reactivity to the tau amino acid sequence of interest.

Figure 4. Tau Antibodies and Immunizing Peptides – Area of Interest amino acids 242-441 of mature sequence.

Table 1.

Antibody	Location	Comments
Tau, pan (DC25)	DRVQSK Location: 347-353	Monoclonal Cat# T-1301 ALZFORUM [25]
TauC antibody	SPQLATLAD EVSASLAK Location: 422-438	Polyclonal [26]
Tau, four-repeat (T4R)	PGGGSVQIVY K Location: 301-311	Polyclonal [26]
Tau, AD (DC11)	KCGSLGNIHHKPGGGQVEVKSEKLDFKDRV QSKIGSLDNITHVPGGGNKKIETHKLTFRE NAKAKTDHGA E Location: 321-391	Monoclonal Cat# T-1303 [26]
Tau, isoform 39 (DC39)	*GDTSPRHLSN VSSTGSIDMV DSPQLATLAD EVSASLAKQG L* Location: 401-441	Monoclonal Cat# T-1304 [26, 27]
Tau (RTA-2)	***KVQIINK KLD*** Location: 273-283	Monoclonal [28]
Tau (RTA-C)	*TLAD EVSASLAKQG L* Location: 426-441	Monoclonal [28]

Anti-Tau, C-terminal	**KNVKSKI GSTENLKHQP GGGKVQIINK KLDLSNVQSK CGS** Location: 253-293	Polyclonal [29]
Tau	AKTDHGA EIVYKSP Location: 384-397	Polyclonal [26]
Anti-Tau antibody	KAKTDHGA EIVYKSPVVS *GDTSPRHLSN VSSTGSIDMV DSPQLATLAD EVSASLAKQG L* Location: 383-441 on mature sequence	Rabbit monoclonal [31]
Anti-TAU	*SSGEPPKSGDRSGYSSPGSPGTPGSRSRTPSLPTPPTR EPKKVAVVRTPPKSPSSAKSRLQTAPVPMPDL**KNVKS KIGSTENLKHQPGGGKVQIINKKLD*** Location: 184-283 (area of interest begins at 242: *LQTA*)	Polyclonal [24] SAB4501823 SIGMA Sigma-Aldrich
Tau 7	Proprietary: 12 aa peptide located somewhere within aa 400-450, closer to middle of protein rather than C-Terminus	Monoclonal [23] MAB2239 Millipore

MSDx Project B - Detection of TAU Protein:

Detection of the tau protein is a multistep process. The blood samples are taken from subjects that have (or may have had) exposure to head injuries, sent to the MSDx lab, and the multistep process occurs with processing the blood, determining the protein concentration with a protein assay, and finally with the detection of tau within the sample using the ELISA assay.

Project B - Step 1: Pre-Analytic Processing of Blood Samples:

PURPOSE:

Separation of blood components using CPT (cell processing tube) to obtain lysates for protein and ELISA analysis.

MATERIALS:

Materials used for the pre-analytic processing of blood samples are approved in the standard operating procedure created by MSDx (document number: LWI2015-01, revision B) [13]. The applicable federal regulations can found in 45 CFR Part 46 (Protection of Human Research Participants) and 29 CFR part 1910.1030 (OSHA Bloodborne Pathogens Standard) [14, 15]. Materials are purchased from companies such as Sigma-Aldrich (RBC lysis buffer and phosphate buffered saline (PBS)), G-Biosciences (protease inhibitor) and Becton Dickinson (4 mL cell processing tubes (CPT)).

PROCEDURE:

Blood samples were sent overnight to MSDx for analysis after collection from the study site in a 4 mL cell preparation tube (CPT) using sodium citrate as the anticoagulant [16, 17]. Upon arrival to MSDx laboratory, proper receiving and handling was conducted as per standard operating procedures by MSDx (documented date received, condition, and sample identifier). Once documented, blood tubes received were centrifuged for 30 minutes on high at 3,000 RPM to form a Ficoll gradient within the tube separating the plasma from the PBMCs and RBCs/granulocytes. Figure 5a demonstrates CPT before and after centrifugation.

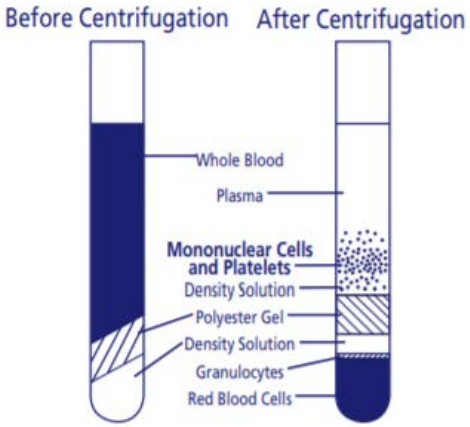

Figure 5a: Cell Processing Tube [73].

After centrifugation, 5 aliquots each containing 100 mcL of plasma was harvested from the upper portion of the tube without disturbing the ficoll layer. The 5 aliquots were frozen (-80°C) for other analyses. The remaining fluid in the CPT tube was poured out into a 50mL conical tube and 1x PBS (pH 7.4) was added to fill the tube (~40-45 mL) and then re-centrifuged for 15 minutes at 1200 rpm. After the second round of centrifugation, a pellet formed at the bottom of the conical tube containing the red and white blood cells. The supernatant was aspirated (vacuum suction) and discarded leaving the pellet undisturbed at the bottom of the conical tube (see figure 5b). Immediately, 3 mL of the red blood cell (RBC) lysis buffer was added and gently vortexed to re-suspend the pellet within the buffer to break up the RBCs contained in the pellet. The pellet was incubated at room temperature for 5 minutes allowing the buffer to break up the RBCs. This first lysis to take place breaks up any residual the reticulocytes (RBCs). Next, 1x PBS (pH 7.4) was added to fill the conical tube (~ up to 45 mL) and centrifuged for 15 minutes at 300 RCF (1200 RPM). The supernatant was once again aspirated (vacuuming up the RBCs that were broken apart) leaving the pellet containing white blood cells undisturbed at the bottom of the conical tube. The pellet was suspended in 125 mcL of deionized water (DI) containing 0.1% sodium azide to break up the white blood cells allowing the debris contained within them to be accessible. After incubating at room temperature for 5 minutes, 2.5 mcL of a protease inhibitor (enzyme) and 125 mcL of carbonate buffer (pH: 9.2, sample becomes more alkaline) was added to the conical containing the lysate (lysis of white blood cells in deionized water). The PBMC lysate was then aliquoted into 5 separate polypropylene microcentrifuge tubes containing 50 mcL each and stored at (-80°C) for future protein assays.

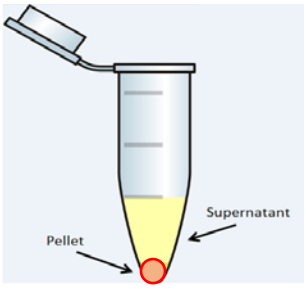

Figure 5b. Example of pellet and supernatant in a polypropylene microcentrifuge tubes (actual procedural aspiration occurs with supernatant and pellet in a conical tube) [18].

Project B - Step 2: Protein Assay:

PURPOSE:

Assess protein content of the lysates (lysis of white blood cells) collected from blood samples from the pre-analytical processing steps that were previously stored in the (-80°C) freezer.

MATERIALS:

This procedure was completed with materials listed in the standard operating procedure approved by MSDx (document number: LWI2016-05A, revision A). Specific reagents and

equipment is from Bio-Rad (coomassie blue, semi-micro cuvettes, spectrophotometer), Sigma Aldrich (bovine serum albumin (BSA)), and Thermo Scientific (1 mL test tubes). The peripheral blood mononuclear cell (PBMC) lysates are from step 1 above (SOP LWI2015 - 01 Revision B).

PROCEDURE:

All needed reagents including PBS (phosphate buffered saline - pH 7.4), BSA standard (a protein concentration standard), deionized water (without 0.1% sodium azide), coomassie blue, and prior lysate aliquots from step 1 were brought to room temperature before use. The BSA standards were made first by adding BSA to 12 test tubes (1 mL) diluted with PBS creating duplicates of the following concentrations: 0, 5, 7, 10, 12, 15 mcg/mL in each (duplicated) tube. Coomassie blue, which binds and stains proteins in lysates was added to each test tube. Test tubes were vortexed vigorously and incubated at room temperature for 5-60 minutes. The same procedure was completed for the lysates (without BSA), 5 mL of each lysate sample was diluted into DI water and then mixed with coomassie blue before vigorously vortexed and incubated along with the BSA standard. After incubation, solutions were transferred one at a time into cuvettes (beginning with the smallest concentration first) and the optical density was obtained using a spectrophotometer at a wavelength of 595 nm. With the results from the spectrophotometer, a standard curve was created on a linear scale with the known concentrations plotted on the X-axis and the corresponding optical density (O.D.) on the Y-axis. Based on the linear trendline, R-squared was calculated to document the value greater than 0.95. With the equation created from the protein assay, the sample concentration for ELISA is determined by calculating the X value from substituting the corresponding O.D. into the trendline equation obtained in the standard curve. An example of a plotted standard curve and corresponding equation can be seen below in figure 5c.

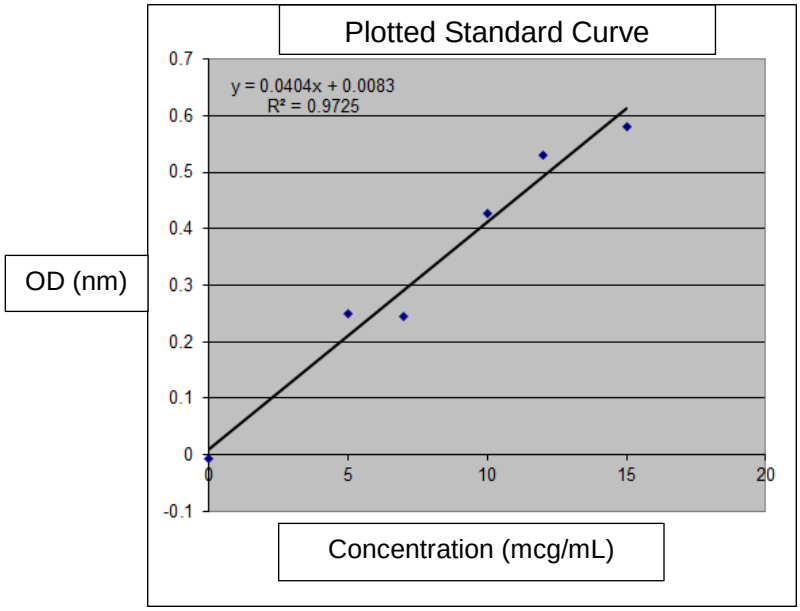

Figure 5c. Example of a plotted standard curve. The equation is used to determine the amount of lysate to use in the ELISA assay (to normalize protein concentration between samples).

Project B - Step 3: Window into the Brain Tau ELISA Assay:

PURPOSE:

Assessing tau protein content of lysates using the indirect enzyme-linked immunosorbent assays (ELISA).

MATERIALS:

The following process is from the standard operating procedure approved by MSDx (document number: LWI2015-04, revision A). Samples are placed into a 96 well clear polystyrene ELISA plate (Thermo Scientific Pierce) and the spectrophotometric ELISA plate reader capable of quantitative absorbance measurement of 450nm (BioTek Elx800). Reagents used for this assay are human Tau-441 isoform (positive control (2N4R) from rPeptide, T-1001-2), PBMC lysate samples previously prepared from the pre-analytical blood processing (SOP LWI2015-01) and BSA (Sigma Aldrich). The primary antibodies used are the polyclonal antibody number 1 (PC1), and monoclonal antibodies 1 and 2 (MC1 & MC2). Further information regarding PC1, MC1 and MC2 is proprietary to MSDx. Secondary antibodies used with the primary antibodies is the anti-rabbit (IgG, peroxidase labeled, Sigma Aldrich A6154), anti-mouse (IgG, peroxidase labeled, Sigma A2554), and Streptavadin (KPL 5140-0006). TMB (visualizing reagent used in ELISA assays, 3,3', 5, 5' - tetramethylbenzidine soluble substrate) is from Calbiochem (613544) and the TMB stop solution is from KPL (50-85-05).

PROCEDURE:

Two concussion samples were ran (C1 & C2) concurrently with two controls, control sample (CS) and Tau 441 - the full tau protein. Based on previous protein assay results from step 3 (SOP LWI2016-05A), the total concentration (diluted in 1x bicarbonate buffer, pH: 9.2) for each lysate was C1: 5 mcg/mL, C2: 5 mcg/mL, CS: 5 mcg/mL, and Tau-441: 1 mcg/mL. 100 mcL of each diluted lysate sample was added in designated triplicate wells in the ELISA plate. The samples were parafilmed and incubated at room temperature for an hour. The 96 well plate was manually washed with DI water 4x to rid of any materials that have not bound to the wells. 300 mcL of blocking solution (1% BSA diluted into PBS) was added and incubated for 2 hours at room temperature. After incubation, the plate was washed 4x with DI water and blotted dry. 100 mcL of the primary antibody was added to each designated well as shown in figure 6 below (no primary antibodies are placed in control wells).

Figure Legend:
C1: Concussion sample 1
C2: Concussion sample 2
CS: Control Subject

96- well clear polystyrene ELISA plate (protein binding)

		1° Ab: PC1 2° Ab: Anti-Rabbit			1° Ab: MC1 2° Ab: Anti-Mouse			1° Ab: MC2 2° Ab: Streptavadin			Control (no 1° ab)		
		1	2	3	4	5	6	7	8	9	2°: Anti-Rabbit 10	2°: Anti-Mouse 11	2°: Streptavadin 12
C1	A	**C1:** 100 mcL is added to each of the 12 wells (1.2 mL total), 35 mcL of lysate diluted into 1.5 mL of 1x bicarbonate buffer											
C2	B	**C2**: 100 mcL is added to each of the 12 wells (1.2 mL total), 29 mcL of lysate diluted into 1.5 mL of 1x bicarbonate buffer											
CS	C	**CS**: 100 mcL is added to each of the 12 wells (1.2 mL total), 46 mcL of lysate diluted into 1.5 mL of 1x bicarbonate buffer											
Tau 441	D	**Tau 441 (full Tau Protein)**: 100 mcL is added to each of the 12 wells (1.2 mL total), 15 mcL of lysate diluted into 1.5 mL of 1x bicarbonate buffer											

Figure 6. ELISA plate set-up

Once primary antibody was added, the 96 well plate was parafilmed and incubated at room temperature for 2 hours. Plate was then wasted with DI water 4x to rid of any antibodies that were not bound to the lysates. 100 mcL of the HRP-labeled secondary antibody was added to each designated well as shown in figure 6 above (including the control wells). The plate was then parafilmed and incubated at room temperature for 1 hour. After incubation, the plate was washed again 4x with DI water. 100 mcL of TMB (soluble substrate) was added to each well and incubated at room temperature for up to 1 hour before the TMB stop solution was added. The TMB stop solution was incubated at room temperature for 5 minutes (allowed to equilibrate) and then read in the spectrophotometer at an optical density of 450 nm within 30 minutes. Results are shown in figures 7A, 7B, and 8 below.

Project B - Detection of Tau Protein Results:

Figure Legend:
C1: Concussion sample 1
C2: Concussion sample 2
CS: Control Subject

O.D. 450nm		1°PC1			1°MC1			1°MC2			Control (no 1° AB)		
		1	2	3	4	5	6	7	8	9	10	11	12
C1	A	0.466	0.431	0.399	0.068	0.062	0.059	0.235	0.258	0.241	0.059	0.079	0.086
C2	B	0.421	0.418	0.401	0.069	0.057	0.062	0.274	0.313	0.334	0.083	0.061	0.098
CS	C	0.39	0.369	0.396	0.064	0.066	0.071	0.202	0.172	0.183	0.061	0.067	0.066
Tau 441 (pos control)	D	1.907	1.689	1.748	0.302	0.252	0.229	3.38	3.053	3.118	0.071	0.08	0.089

Figure 7A: Tau indirect ELISA assay results from spectrophotometer.

Figure Legend:
C1: Concussion sample 1
C2: Concussion sample 2
CS: Control Subject

	Averages	PC1	MC1	MC2	Control
C1	A	0.432	0.063	0.2447	0.074667
C2	B	0.4133	0.06267	0.307	0.080667
CS	C	0.385	0.067	0.1857	0.064667
Tau 441	D	1.7813	0.261	3.1837	0.08

Figure 7B: Averages of ELISA results for Tau protein (the higher the average the greater the amount of protein detected). Readings taken at OD450nm

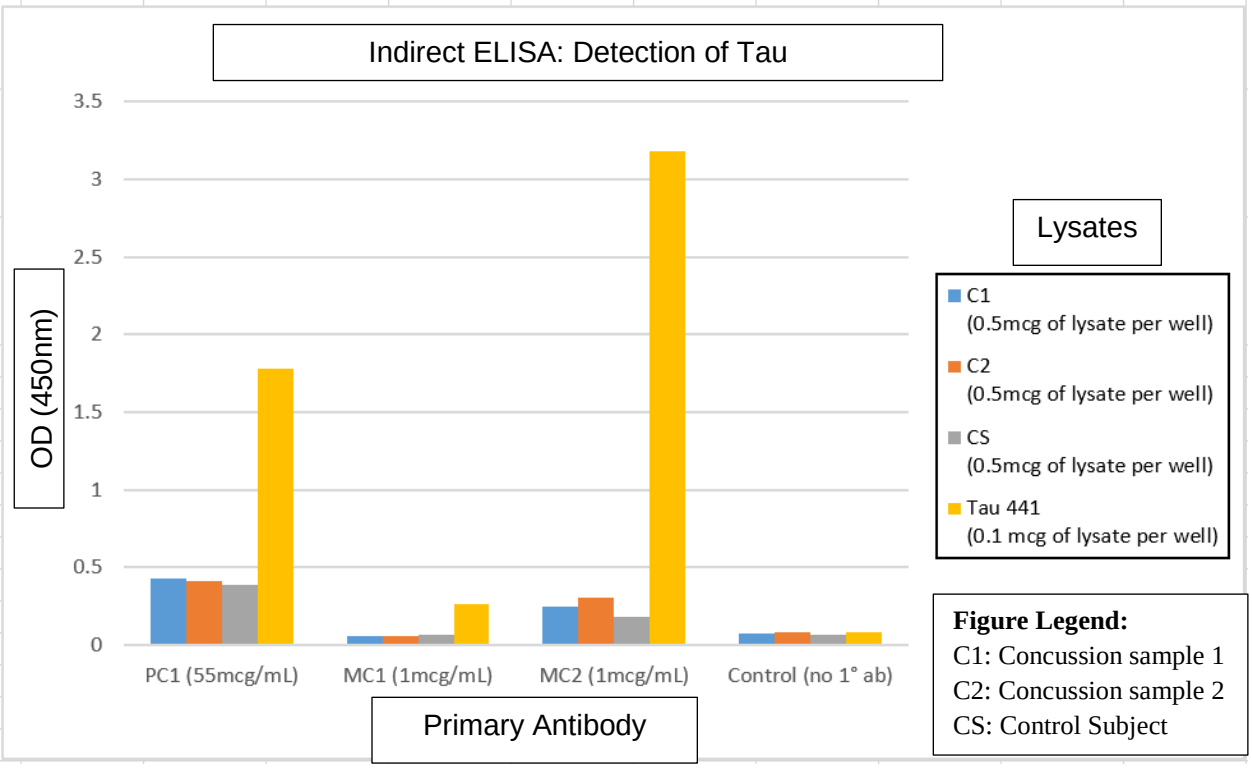

Figure 8: Lysates from concussion subjects (C1 and C2) and the healthy control (CS) were diluted to a protein concentration of 5 mcg/mL. The ELISA plate wells were coated with 100 mcL with each lysate giving 0.5mcg of protein per well. Reactivity of the lysates with the polyclonal (PC1) and monoclonal antibodies (MC1 & MC2) are evident.

Project A & B Conclusion:

The literature search was successful in finding a number of sources in anti-tau antibodies with the majority having known epitope amino acid sequences. In some cases the epitope wasn't entirely defined but an immunizing peptide sequence was available (see Tau 7 in table 1). This knowledge could enable identification of antibody pairs for a future sandwich assay but will require empirical testing to determine whether or not they detect tau fragments within the phagocytic lysate.

As for the indirect ELISA demonstration, based on the results (figure 8), in combination with Tau-441, the optical density results from PC1, MC1 and MC2 all demonstrate positive binding interactions. Tau-441 is a recombinant protein demonstrating the full length sequence of tau so reactivity with the polyclonal and monoclonal antibodies to tau are expected. However, the weaker reactivity seen with MC1 may reflect specificity for a conformational determinant that may not be present in recombinant tau protein or may be disrupted by adhesion of tau protein to the plastic surface. Another possibility for the lack of reactivity is that the fragmentation process may cut through an epitope sequence leaving a partial epitope in which the antibody recognizes with reduced affinity. MSDx has found that PC1 reacts strongly with tau-441 at a 1:32000 dilution but at a 1:32000 dilution, it doesn't show positive binding to

pathological lysates containing tau. This implies that a minor antibody component of the polyclonal mix is responsible for detecting a relevant tau peptide that isn't detected by the other antibodies that are likely detecting other epitopes. The reactivity of CS with PC1 gives a high background at a 1:100 working dilution. Presumably, the high background seen on the control lysate (CS) is due to nonspecific binding of the high levels of antibody that do not detect relevant peptides within the macrophage lysate. The results in figures 7A, 7B, and 8 also show the comparative binding of one polyclonal antibody (PC1) versus two monoclonal antibodies (MC1 & MC2). Of the two monoclonal antibodies, one has weak binding to lysates and the other demonstrates strong binding to lysates containing tau fragments. However, it should be noted that this ELISA assay alone has not shown definitively that these lysates contain tau fragments. The assumption that they contain tau is being made because the lysate is derived from mononuclear blood cells, but further protein analysis remains. The control wells, with no primary antibody, compared to CS wells, greatly suggests that the optical density (OD) seen within CS suggests high non-specific binding of the primary antibody. In the absence of primary antibody the OD is very low, further emphasizing that the polyclonal antibody is the source of increased non-specific binding of antibody epitopes not represented within the lysate. One thing that wasn't addressed is that the standard curves with recombinant tau were all performed in PBS and doesn't account for any matrix effects due to the other proteins (or components) within the lysate. To control for this lysates from normal controls should be spiked with recombinant tau and assayed according to the usual protocol; this will allow the assessment of how the matrix affects the measured optical density.

PROJECT A & B FUTURE CONSIDERATIONS:

For project A, MSDx will need to commercially test the monoclonal antibodies for reactivity with lysates and select working pairs for sandwich assay development. First step would be to test an antibody in the current first generation assay test and if positive in detection of tau (with known lysate samples to demonstrate degeneration) then the antibody would be a candidate for sandwich assay reagents.

The next steps to be completed for project B are to epitope map the activity within the PC1 antibody and make a specific monoclonal to that epitope. Completing that task should remove the nonspecific background issues. In addition, it would be prudent to control for matrix effects of the lysates using normal controls (CS) which should be spiked with varying concentrations of recombinant tau and assayed according to the usual protocol this will allow the assessment of how the matrix affects the measured optical density.

CHAPTER 2: Comparison between the various microsampling devices including current advancements to address the hematocrit bias.

ABSTRACT

Pharos Dx uses liquid-chromatography mass spectrometry (LC/MS) for the commercialization of existing, emerging and future human and animal diagnostic testing in support of the medical, scientific and research communities. Pharos Dx is looking for innovative ways to assist healthcare practitioners potentially introducing microsampling to their services to address an unmet need with venipuncture. A literature search and review evaluating the advantages and disadvantages of microsampling within current published work was conducted. Microsampling tools were compared assessing their current methods (validation and verification) and procedures used including the precision and accuracy of tests, user friendliness, and effectiveness against the hematocrit bias. With the various techniques available, two front runner devices, dried blood spot cards and volumetric absorptive devices such as Mitra® were found to outperform the other microsampling options.

INTRODUCTION:

Pharos Diagnostics (Pharos Dx), is a high-complexity diagnostics laboratory providing much needed services to clinicians, researchers, veterinarians, and drug-development companies [31, 33]. Established in Tucson in 2016, the CLIA (clinical laboratory improvement amendment) certified laboratory, along with their experienced team, uses high performance liquid chromatography (HPLC) separation method followed with mass spectrometry (mass spec) to perform molecular analysis [32, 34]. Mass spec works by measuring a mass-to-change ratio of ions therefore identifying and quantifying molecules based on their masses through an electric or magnetic field [32, 34, 35]. As described by ThermoFisher Scientific, liquid chromatography-mass spectrometry (LC/MS) is commonly used for analysis in sensitive biological fluids such as blood (and other thermally unstable or nonvolatile molecules) [35]. This type of technology has given Pharos Dx a competitive edge as they continue to increase the services they provide to humans and animals (now providing testing for Valley Fever) [32, 36].

The goal of the internship was to conduct a thorough literature review of published microsampling devices and techniques. The highly qualified team at Pharos Dx would like to introduce microsampling for humans and veterinarians as part of their offered services in the future. Providing microsampling services would address an unmet need, allowing sampling of biological fluids (i.e. blood) at the convenience of the patient's home with reduced pain and stress.

Microsampling is a technique that utilizes a much smaller volume of blood and generally requires only 10-20 mcL to use for detection of an analyte of interest. Microsampling works with fluids collected from the prick of a finger/heel in humans and from a prick of the tail or ears in animals (mice, dogs, cats, etc) [37]. With a single prick, a drop of pooled blood forms at the surface and is collected onto an absorbent surface. That small drop would contain enough material to provide diagnostic information for a variety of analytes (hormones, vitamins, drugs, etc). Venipuncture, the more conventional way of obtaining blood, requires more time and

equipment as well as pain and stress to the subject/patient during the draw. Figure 9 below provides a visual of the differences between conventional venipuncture and two microsampling devices that will be discussed within this paper.

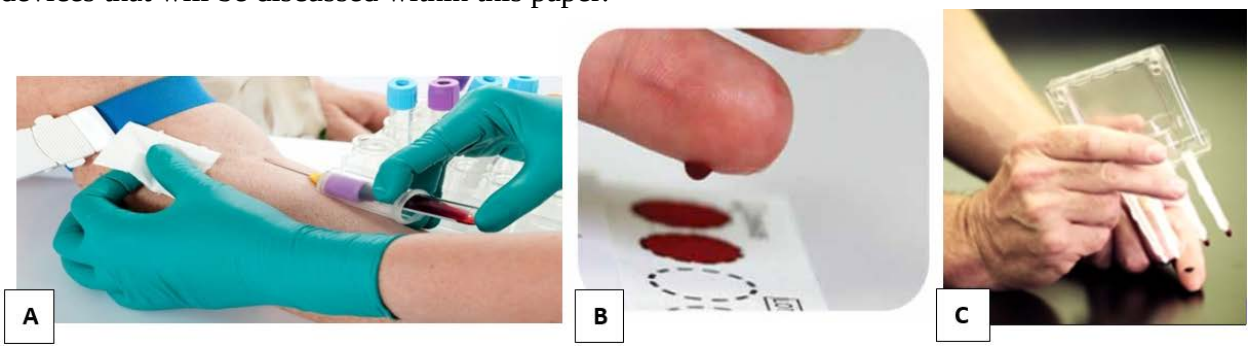

Figure 9. Venipuncture (A) requires a trained professional to be involved in the collection of blood. More materials and equipment are needed for this procedure and also require a larger volume of blood to be collected. Dried blood spot cards (B) and volumetric absorptive microsampling devices (VAMS) such as Mitra® (C) can be done by the patient, in the comfort of their own home, collecting a much smaller volume that is still accurate for diagnoses [37, 38, 39, 54].

Conventional methods, often requiring a larger volume of blood, can be a problem for people with small or inaccessible veins due to age or illness or to those that have a great fear of needles. Once collected, vials containing the blood must be transported by a trained medical professional, would require refrigeration (depending on the collection tube and sample), and can pose as a biohazard during transportation if broken. As venipuncture requires extra time, equipment, processing, and shipping requirements, this is a problem in locations that are not easy to reach by medical personnel such as the case in very rural communities. Microsampling, however, can be done at home by the patient themselves with a prick of their finger. Once the blood is collected, the sample is air dried (rendering infectious pathogens to be inactivated) before being placed in the mail and shipped to the lab via normal mail carrier. This option is not only attractive for the convenience to patients, but also for effectively implementing the 3R's (reduction, replacement, and refinement) as an alternative for animal testing to reduce pain and stress for lab animals and in veterinary diagnostics [67, 68]. At the conclusion of the internship, information such as research articles, contact information, and suggested procedures were compiled and given to Pharos Dx for future review and use. This report is a summary of the information researched during the internship period.

METHODS:

The literature search was conducted primarily through use of the internet with research articles found on the University of Arizona library database, science direct, etc. Devices researched were dried blood spot cards (DBS) and volumetric absorptive microsampling (VAMS) devices. References within the articles found were also used. Companies and brands researched included HemaXis [53], Neoteryx [54], and Novilytic [55]. Email correspondence and skype meetings took place with representatives from HemaXis, Neoteryx and advocates for DBS cards.

RESEARCH REGARDING MICROSAMPLING ISSUES:

With such a useful tool that would eliminate much of the inconvenience pertaining to venipuncture, there is still a big issue that is holding the success of microsampling back; the overall hematocrit. The hematocrit is the volume (ratio) of red blood cells (RBCs) in the total volume of whole blood (containing RBCs, WBCs, platelets and plasma) [44]. Essentially, it is the percent of RBCs within the blood and all of the included components. Hematocrit (hct) can naturally vary depending on the subjects' age, gender, and health but the general reference ranges are 40-54% in men and 36-48% in females [41, 44]. Figure 10 below demonstrates how the amount of RBCs are affected with a varying hematocrits within the vein.

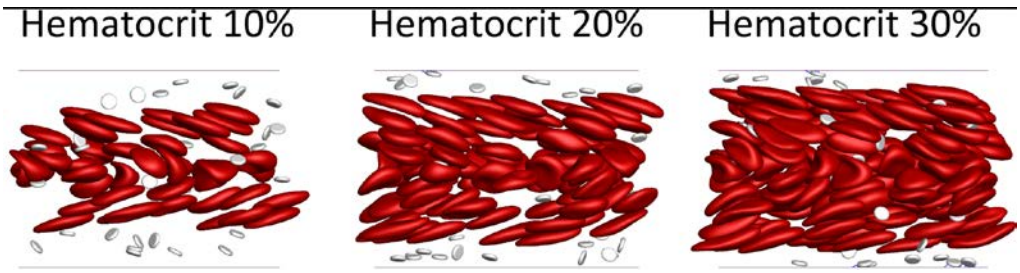

Figure 10. The hematocrit indicates the difference between having a low hematocrit (10%), less RBCs, and a high hematocrit (30%), more RBCs which in turn can affect the spreading of the blood on dried blood spot filter paper [44].

Whole blood contains plasma, white blood cells (WBCs), platelets, RBCs and a variety of other components to make up the total volume of blood drawn. The hematocrit is the percent RBCs found in the blood, as can been seen in figure 10 and 11. Figure 11 below demonstrates how a low or high hematocrit would be reflected after centrifugation in a blood collection tube (normal hematocrit reference ranges can very slightly according to hospital or health institution).

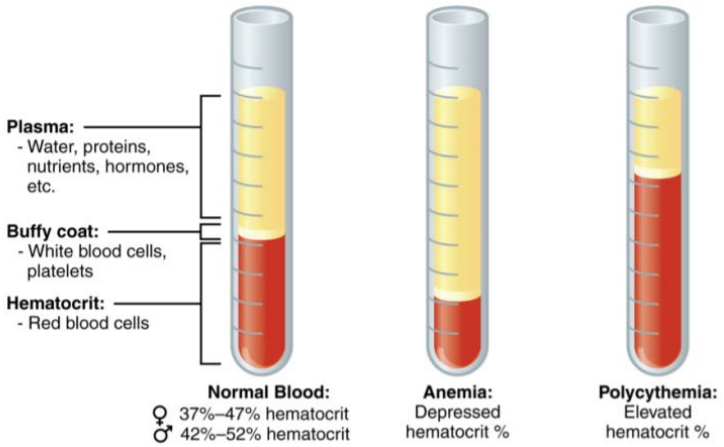

Figure 11. Example of how hematocrit levels would affect the volume of RBCs collected in blood tubes (anemia: low hematocrit, polycythemia: high hematocrit) [44].

The hematocrit remains to be the most widely known problem in dealing with microsampling. Current research addresses novel techniques used to overcome the problem with the hematocrit including measuring other analytes as indicators for the hematocrit, to creating new microsampling devices altogether. This research paper will address the two most common

approaches, dried blood spot cards (DBS) and the newly developed potassium algorithm and volumetric absorptive microsampling (VAMS) devices such as Mitra®.

DRIED BLOOD SPOT CARDS (DBS):

Dried blood spot (DBS) cards were originally introduced in 1963 by Robert Guthrie for phenylketonuria (PKU) screening in neonates as a new method to explore [45]. The two most common and FDA approved class II filter papers used are Whatman 903 and Ahlstrom 226 filter paper which are both made from high-purity cotton linters [56, 57, 58, 60]. The filter paper is manufactured to yield accurate and reproducible blood samples according to the Clinical and Laboratory Standards Institute's specifications (NBS01-A6) [56, 57, 58, 60]. The summary of the research published in 1963 concluded that using dried blood spot cards to detect for elevated concentrations of blood phenylalanine that is commonly seen with phenylketonuria provided accurate results and were comparable to other sampling methods used at that time [45]. Even with the FDA approved filter paper, it has been well established that the hematocrit as well as the homogeneity of the sample remains to be a prominent issue dictating the amount of RBCs present and the equal concentration of analytes across the same blood spot [59].

Dried blood spot (DBS) cards have been a major player in the microsampling world and have been used for decades. The normal procedure for DBS cards is to obtain a drop of capillary blood (from finger or heel) and allow the whole blood sample to be absorbed onto filter paper. Once air dried (2-3 hours at ambient temperature), the DBS cards can then be shipped to the laboratory for testing though normal mail carrier. The spots are hole-punched (usually 3mm) and diluted into a solution (deionized water or methanol plus an internal standard, dependent on the analyte of interest) for further testing. Figure 12 below demonstrates an example of how this generalized process occurs.

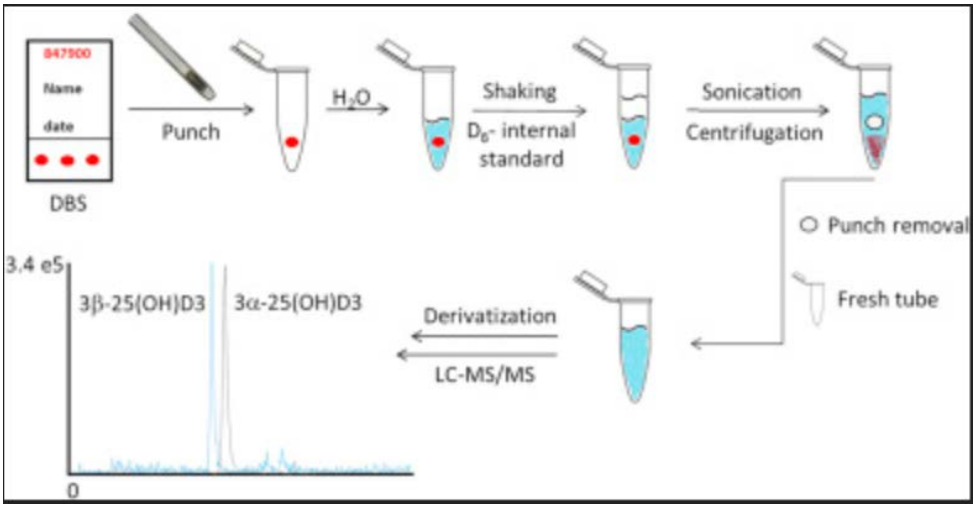

Figure 12. A generalized procedure for extracting dried blood spot punches for use in liquid chromatography mass spectrometry (LC/MS) [48].

The advantages of the dried blood spot process is cost effective as multiple spots can be collected and it can be completed in the home of the patient (by the patient) without a certified phlebotomist. Once blood spots are collected, DBS cards can be shipped via normal mail carrier and be stored at ambient (room) temperature.

As discussed previously, the hematocrit problem remains to be the biggest disadvantage with dried blood spot cards as it is not a fixed volume (non-volumetric) applied to the filter paper. As can be seen with the example of the Whatman 903 card in figure 13 below, without a known volume of blood being applied to the filter paper, a variable amount of blood can be absorbed. The hematocrit determines the viscosity of blood and alters the blood spreading evenly throughout the filter paper, the amount of blood in each 3mm punch will contain different volumes of blood. The homogeneity of the sample is also affected as the concentration of an analyte might vary depending on where the punch is taken. The varying hematocrit and homogeneity of the sample could produce unreliable results (higher/lower values) and affect overall treatment and diagnosis.

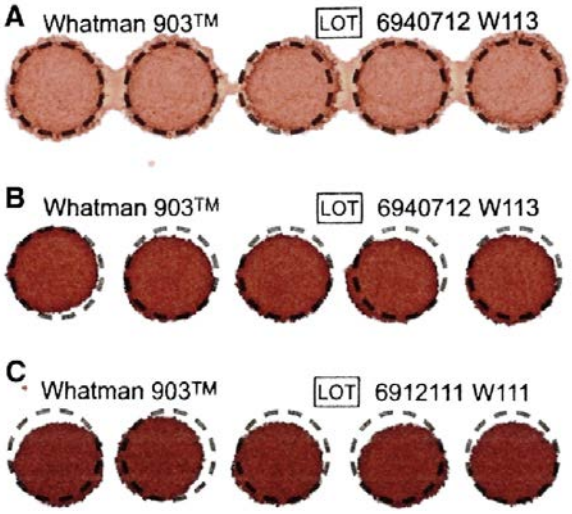

Figure 13. Example using the dried blood spot Whatman 903 card of the various effects of the hematocrit level. **A.** Hct = 18%, **B.** Hct = 35%, **C.** 50% [43].

With the issue of the hematocrit, there have since been a variety of proposed solutions in research to overcome this problem. Many are in agreement that to eliminate the hematocrit issue is to apply a known volume to the DBS card and punch the whole spot out for use in analysis [47, 50]. There are devices available for applying a volumetric spot onto the filter paper for whole-spot analysis, but using those applicators, such as a micropipette, would require a trained professional in a controlled setting to apply the spot and eliminate contamination [47, 50]. Suggestions to address the hematocrit problem while keeping DBS cards available for home use includes a technique using temperature-enhanced flow-through desorption in solid phase extraction and mass spec, presented in 2013 from Ooms *et al* to decrease the effect of the hematocrit and the recovery of drug analytes [47, 49]. Dried plasma spots have also been discussed by multiple researchers that would eliminate the hematocrit by using plasma instead of whole blood for analyte analysis [47]. However, to obtain plasma from whole blood, this would still require venipuncture to retrieve the blood and centrifugation to obtain only the plasma (see figure 11 for reference) which is not ideal for patient home use [47, 51].

However, with this unique idea in mind of using dried plasma spots (DPS) rather than DBS, there is a device created that offers a multi-filtration membrane to separate plasma from whole blood [47, 55]. In 2012, Li *et al* presented research on using “two-layered polymeric membrane” cards to obtain plasma for analysis of the drug guanfacine (used to treat high blood pressure and ADHD) using a semi-automated procedure with LC/MS/MS [61]. In addition, the

Novilytic company has been working on developing the Noviplex plasma prep cards (figure 14 below) offering the single plasma collection volume (~25 mcL of whole blood required to obtain ~2.5 mcL of plasma) [55].

Figure 14. Noviplex (Novilytic) Plasma Prep Card [55, 62]

A blood drop is applied directly to the noviplex card within the target area, absorbed and filtered through a membrane separating RBCs (by size) collecting only plasma as the end result. The filtration process is due to capillary action pulling the fluid through the card and using size filtration to separate the RBCs from the plasma. After the 3 minute drying time, the upper layer containing the RBCs can be pulled off and discarded exposing only the plasma for extraction. Figure 15 below visualizes this filtration process.

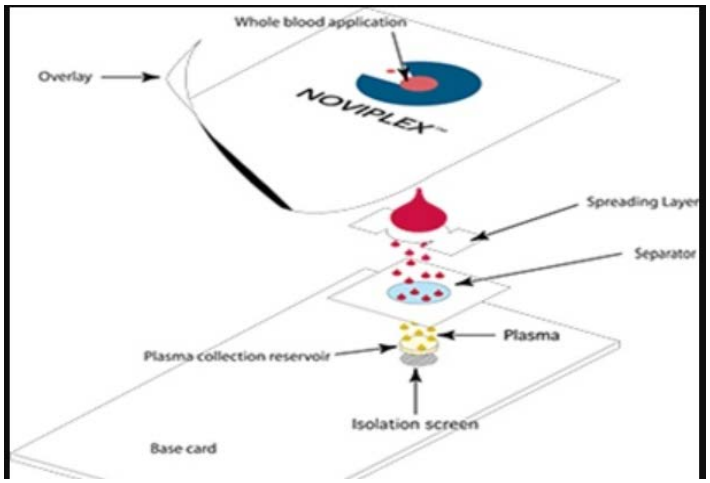

Figure 15. Filtration process for Noviplex Single (uno) plasma collection card [55, 62]

Novilytic also came out with a duo card option which is designed to collect more than the standard card (~60 mcL of whole blood needed to collect ~7.6 mcL of plasma) and is marketed to be compatible with LC/MS [55, 63]. It was also featured in the Purdue University Research Foundation News as a class 1 medical device for winning the 2014 R&D 100 Award due to the novel way of separating plasma without the need for any laboratory equipment [65]. Disadvantages of the Noviplex card include a much smaller volume size (up to ~7.6 mcL max) which can be a disadvantage for some analytes that need a larger volume for analysis. Obtaining a substantial amount of the cards for use and testing can be expensive for the company ($10-$14.50 per card) as well [55].

Another promising solution is using another analyte as an indicator of the hematocrit within the blood spot. According to Capiau *et al*, it was discovered that the hematocrit value could be predicted using an endogenous compound such as Potassium (K+) [46, 47]. Capiau *et al* found within their research for potassium levels in 3mm extractions from DBS punches to be a reliable indicator as it correlates with the amount of RBCs present, is universal, and it is stable over time leading to be chosen as a marker for the hematocrit [52]. The published report describes the fully validated procedure using potassium levels from 3 mm DBS extracts (tested with 30-70 mcL of various elution solvents such as PBS), along with a routine chemical analyzer (Roche Cobas 8000) to predict the hematocrit of samples ranging from 19%-63% with accuracy and precision [46, 47, 52]. This novel technique was demonstrated using the developed algorithm (potassium based algorithm) to correct for the hematocrit when testing for quantitative analysis of caffeine and a major metabolite, paraxanthine, in DBS [64]. The potassium algorithm used for the quantitative analysis for caffeine in DBS was also compared using volumetric absorptive microsampling (VAMS) which will be discussed next in this paper.

VOLUMETRIC ABSORPTIVE MICROSAMPLING (VAMS):

To address the hematocrit problem that is commonly seen in the non-volumetric dried blood spot cards, a novel device known as Mitra® offered by Neoteryx is another promising contender. Mitra® is an FDA approved class 1 microsampling device that collects a fixed volume of blood allowing patients to self-collect at home, air dry, and ship back to the lab as one would with DBS cards [54]. Neoteryx offers multiple "user friendly" formats enabling 10, 20 or 30 mcL of whole blood per finger/heel prick to be absorbed. Figure 16 demonstrates how the device is held at a 45 degree angle allowing the blood to be absorbed controlled by polymeric substrate through capillary action within the porous and hydrophilic surface of the Mitra® tip [54, 59].

Figure 16. The application of Mitra® microsampling tool within the purple cartridge for ease of handling and use [54, 59].

Once the tip is saturated, the whole tip extracted and used for either immediate analysis or stored for days to months depending on the stability of the analyte within the sample [59]. An issue with VAMS is the way the blood is collected and the complications involved with incorrect application. In two studies in rats, researchers demonstrated different ways to obtain blood from the rats' tail, one way which resulted in oversaturation of the tip and another way to overcome that issue. This can be seen in figure 17 image A and B below.

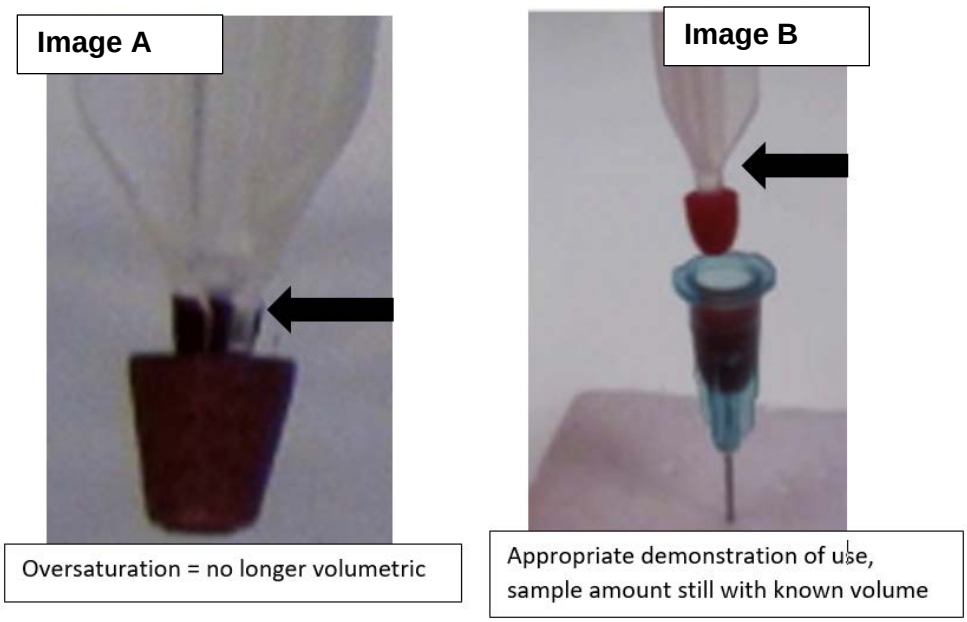

Figure 17. Image A demonstrates increased blood flow at a faster rate than can be absorbed leads to more volume collected within the shoulder of the tip, which therefore alters the total volume, the volume is no longer known. **Image B** demonstrates a way to control the bleeding rate to solve the oversaturation issue by using a 23G needle allowing the VAMS device to pull up the necessary amount needed without absorbing more than allowed (image modified from original data and photos [66, 67]).

Figure 17 is from a toxicokinetic study in rats published in 2015 addressing the issue with collection of blood with VAMS. A butterfly infusion set was used to collect blood from a rat tail but as the flow of blood was greater than rate absorbed by the VAMS device, a pool of blood aggregated on the tip shoulder leading to non-volumetric collection (figure 17 image A) [59, 66]. It was thought that the increased flow rate was due to the administration of a particular drug utilized in this study, as a result, other methods were adapted. Using a 23G needle (figure 17 image B) allowed the VAMS device to be applied within the luer lock controlling the flow rate of blood. The same 23G needle can be used, as tested by another study published by Thiry *et al* to effectively puncture the skin and allowing the VAMS device to be directly applied to the blood drop on the tail [59, 66, 67].

In addition, a cross-laboratory study was done with 6 laboratories using VAMS devices to test 6 whole blood samples containing different hematocrits (20%, 45%, 65%) [59, 69]. The average was taken and compared across the 6 laboratories which can be seen in figure 18.

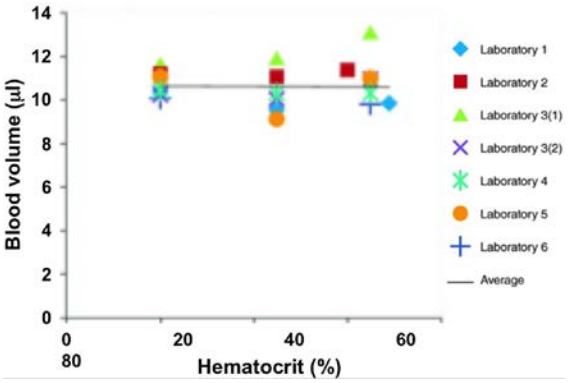

Figure 18. Average hematocrit values of control blood samples tested at 6 different laboratories using VAMS devices (photo taken from [59], original data from [69]).

The authors of the cross-laboratory study concluded the effectiveness of using the VAMS devices as the variability within each lab was within acceptable validation ranges for quantitative bioanalytical methods (calculated CV: 8.7%) [69]. The authors pointed out that there is indeed a greater variance between the minimum and maximum blood volumes absorbed (9.1 to 13.1 mcL) in which there could be more than one explanation to account for this [69]. Further investigation to the proper handling of the tips and procedure during collection and processing within each laboratory could be refined to address any issues with the variation of results within each lab [59, 69]. Figure 18 also demonstrates another issue pointed out by researchers (discussed further in this paper) as there seems to be low recovery with high hematocrit and high recovery with low hematocrit.

COMPARISION BETWEEN DBS AND VAMS

In 2015, a comparative study was done by Kesel *et al* comparing the effects of the hematocrit on extraction of caffeine and paraxanthine from blood collection tubes, VAMS, and standard DBS [70]. The procedure was done with a fully validated LC/MS/MS method for analysis of caffeine and paraxanthine in VAMS, DBS, and whole blood [70]. This comparative study was described by the authors as the first “in-human” study done where analytes collected by VAMS are then compared to the standard DBS or the traditional whole blood venipuncture [59, 70]. For the study, 96 whole blood patient samples with hematocrits ranging from 21% to 50% were collected. The hematocrit of each patient was first established using a Sysmex XE-500 hematology analyzer before collecting blood for comparison with VAMS, DBS and blood tubes [70]. Figure 19 below shows the results of this study [59, 70].

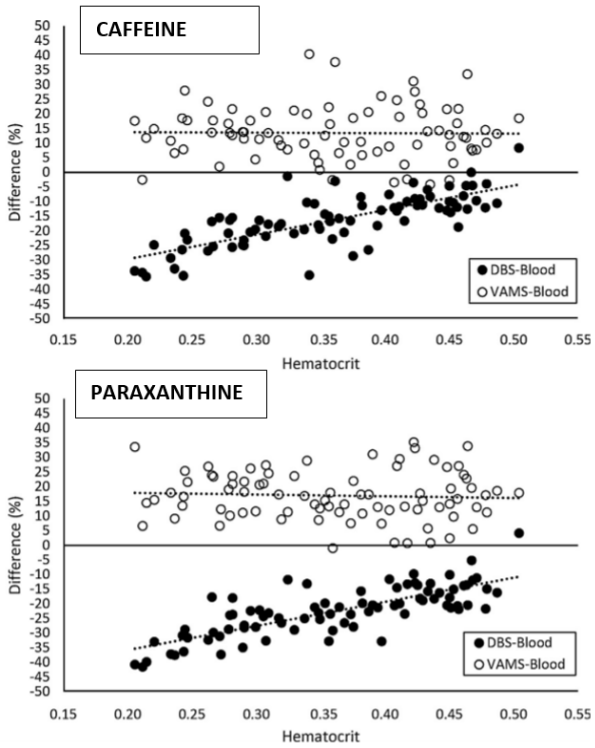

Figure 19. Graphical differences (%) between the three methods of collection. The solid line represents the whole blood concentration (venipuncture) and the dotted lines represent linear regression lines (DBS and VAMS) [59, 70].

Figure 19 demonstrates that there is indeed the hematocrit effect when using dried blood spot samples (not corrected with the potassium algorithm in this published comparative study). The VAMS and whole blood concentrations did not demonstrate significant change in reference to the hematocrit over patient samples [70]. However, it was noted in the research there was an issue with the recovery of the analyte with the hematocrit-independent extraction that isn't seen in the DBS cards (also observed in a different study seen in figure 18). But it was indicated that the recovery could be resolved with other extraction methods, for example, use of a sonicator [59, 70]. It was also pointed out that the Bland-Altman plot used in this study (figure 19) does show that VAMS has a consistent positive bias over the whole blood values (solid line) demonstrating analyte concentrations collected using VAMS is overestimated the concentrations from the collection of whole blood. The study poses this could be due to the different matrices used (liquid whole blood vs dried VAMs sample), and difference in extraction procedures [59, 70]. The authors pointed out this is in support of previous research using VAMS in artificial (non-patient) samples therefore indicating that VAMS does indeed collect the correct volume of blood independent of the hematocrit [59, 70]. More comparative studies with various analytes should be done to produce validated VAMS procedures, but ultimately, VAMS seems to effectively eliminate the effect of the hematocrit [70].

In addition to the well-known VAMS hydrophilic tip and applicator, a novel device combining the volumetric aspects of VAMS and the easy spotting technique of the DBS was combined into a new microsampling tool [70]. The new microfluidic-based sampling procedure was first described in a 2015 Analytical Chemistry publication as a new way to address the

hematocrit effect by volumetrically applying blood to DBS filter paper [70, 71]. Figure 20 demonstrates this foldable support system.

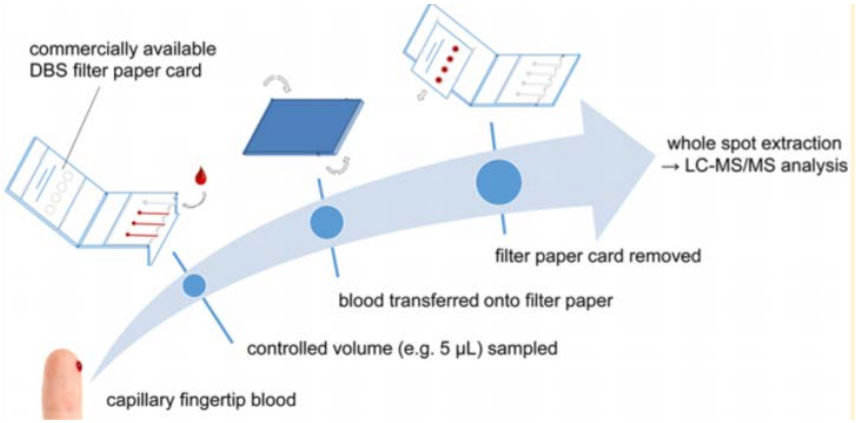

Figure 20. The new microfluidic-based sampling procedure offering DBS card on one side of the foldable structure and sized capillaries allowing a specific amount of whole blood to be absorbed on the other. The blood is supplied to the capillary side, a controlled and set amount of volume is absorbed, the device is closed and the blood is added to the DBS card. The whole blood spot is punched out and used for analysis [71].

Currently marketed by HemaXis, a company located in Switzerland, the FDA class I medical device is designed to collect 5 - 10 mcL of whole blood for analyte analysis. The 2015 article used the device to test its abilities by quantifying caffeine and paraxanthine to see if the hematocrit made a difference. The hematocrit levels ranged between 26% and 62%, the interspot precision from the application of the sized capillaries to the DBS card was deemed to be less than 9% which similar to being manually applied with a pipette. Ultimately, it was concluded to have no effect on the hematocrit for this particular study [71]. Although this novel product demonstrates a positive and promising direction on microsampling more studies need to be published in order for a definite stance on the effectiveness of this device on the hematocrit and its applications for at home sampling (it has not been established if this device has been approved for home use).

In regards to at home sampling, an additional study published in 2016 was done comparing the at home use for monitoring of the HcA_{1c} in diabetes patients (adults and children) using standard DBS cards and VAMS. In this study, venous/capillary samples were taken for immediate HcA_{1c} analysis in adults and children with diabetes in a controlled hospital facility. DBS cards and VAMS were collected at home, air dried, and sent back to the lab for analysis (both were dried samples). During the initial collection of venous/capillary blood for immediate analysis, VAMS was concurrently collected in 25 pediatric patients and were analyzed immediately without drying utilizing another technique known as “wet VAMS” [59, 72]. The results concluded that the dried DBS and dried VAMS sent into the lab from at home sampling (average time before analysis of dried samples = 3 days) showed poor agreement with the venous/capillary samples collected in the controlled hospital facility. However, there was an excellent comparison between the “wet VAMS” sample and the venous/capillary sample collected for the HcA_{1c} results showing strong agreement between the two techniques [72]. Further research needs to be conducted for this difference as there could be an issue with the stability and storage of the specific analyte of interest (in this case HcA_{1c}) [59, 72]. It would also

be beneficial in future studies to conduct the same comparison but using the potassium algorithm to predict the hematocrit from the dried blood spot cards collected at home. In addition to this study, supplemental data was collected as patients were given a questionnaire regarding the at home use between DBS and VAMS devices. Patients agreed the microsampling tools were convenient and easy to use. However, the VAMS device stood out as the preferred method of use among patients which can be seen in figure 21 below.

	Preferred sampling technique				
	Traditional blood sampling	DBS	VAMS	DBS and VAMS	No opinion
Adults	21%	15%	44%	10%	10%
Children	12%	14%	64%	10%	0%
All	16%	14%	56%	10%	4%

Figure 21. Patient responses to questionnaire regarding sampling methods (venous/capillary, DBS, & VAMS) Image modified from [72].

CONCLUSION:

In conclusion, conventional venipuncture requires a trained professional (phlebotomist, nurse, etc) to collect a large volume of blood in a controlled environment. Microsampling offers less stress and ease allowing patients to self-collect at home with a finger prick providing less pain and coordination for collection. The sample is air dried and then shipped via normal mail carrier (hazardous effects are inactivated after air drying) to the lab for analysis. This option allows for the convenience of the collection as well as the ideal device to use for hard to reach veins due to age or illness.

Two microsampling devices, one volumetric (VAMS) and the other non-volumetric (DBS), are both promising options with numerous publications discussing their procedural advantages and disadvantages. However, the standard dried blood spot card has been around for much longer than the volumetric absorptive microsampling devices. The issue of the hematocrit remained the reason why the DBS was not progressing in the microsampling world. To adjust that, a potassium algorithm was developed to use as a marker to predict the hematocrit using a chemical analyzer. In addition, there are many studies published for automated extraction procedures for DBS, which is useful in a large laboratory setting where there is a high volume of testing that is occurring. Mitra® (VAMS device) on the other hand, was created to address the hematocrit issue (before the potassium algorithm was established). While VAMS has been shown to work independent of the hematocrit, studies have confirmed extra attention is needed for the recovery of the analyte of interest (demonstrated to be mostly resolved using a sonicator). And while there are automated procedures for DBS analysis, more research needs to be done to find validated automated procedures for VAMS devices. An additional factor to consider in the decision making process of which device to add to Pharos Dx testing services would be overall cost to run the procedure (chemical analyzer and/or sonicator).

However, at the conclusion of this internship, it was my recommendation to Pharos Dx to begin microsampling testing with validated procedures for DBS using the potassium algorithm to predict the hematocrit. The second option would be to begin testing using a VAMS device along with sonication for optimal analyte recovery.

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