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Tear Component Interference In Bead-Based Biomarker Assays Using Magnetic Beads

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TEAR COMPONENT INTERFERENCE IN BEAD-BASED BIOMARKER ASSAYS USING MAGNETIC BEADS

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ABSTRACT

Cytokines in tears can be biomarkers for ocular inflammation in dry eye subjects. Multiplex bead-based assays measure the concentration of multiple cytokines in a single tear sample. Bio-Rad® polystyrene bead-based assay kits have provided acceptable results for tear samples in this lab. Recently, Bio-Rad decided to replace polystyrene beads with magnetic beads. In earlier experiments in this laboratory, magnetic bead-based assays did not produce results equivalent to polystyrene bead-based assays. The purpose of this investigation was to modify the magnetic bead-based tear cytokine assay to produce equivalent results to those obtained with the polystyrene bead-based assay. The modifications included running numerous bead-based assays that used different wash systems, plate types, and buffers in order to produce the least interference. Manual and automatic wash systems produced similar results but the automatic was superior because of its automation. The magnetic beads on the plastic plate produced more bead aggregation and worse linearity of dilution than on the filter plate. Tears were stored in proprietary and serum-containing buffers, such as Teknova assay buffer, Millipore assay buffer, and Brookwood Biomedical serum buffer, in an attempt to reduce interference in bead-based tear assays. Overall, the results showed the automatic wash system, the filter plate, and Teknova assay buffer with anti-protease to be optimal for tear bead-based assays. Using these optimal conditions, the final comparison of polystyrene and magnetic bead-based

assays showed similar linearity of dilution, which is crucial for small tear samples of dry eye subjects, and better spike-recovery, which measures interference from tear matrix effects, with polystyrene bead-based assays. The results of the final comparison made the polystyrene assay more reliable for future tear assays. Direct comparisons of the two assay types showed the magnetic bead-based assay to be underestimating the polystyrene bead-based assay results. The conclusion was the magnetic bead-based assay was unable to produce results equivalent to polystyrene bead-based assay results. These results led to the decision to use polystyrene bead kits from a company other than Bio-Rad and explore aggressive buffers to reduce interference for future tear assays.

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LIST OF ABBREVIATIONS

ADDE	aqueous-deficient dry eye
AE	aqueous and evaporative dry eye
AP	anti-protease
APC	antigen presenting cell
BAB	Bio-Plex assay buffer
BBA	bead-based assay
BBS	Brookwood Biomedical serum buffer
BSA	bovine serum albumin
BSD	Bio-plex human serum standard diluent
CV	coefficient of variation
D	dry eye
DAB	Diaclone standard diluent buffer
DTT	dithiothreitol
EDE	evaporative dry eye
ELISA	enzyme-linked immunosorbent assay
FBS	fetal bovine serum
FGF	fibroblast growth factor
G-CSF	granulocyte colony-stimulating factor

GM-CSF	granulocyte macrophage colony-stimulating factor
IFN	interferon
IL	interleukin
IP	interferon-inducible protein
LFU	lacrimal function unit
LLOQ	lower limit of quantification
LN	Lupus non dry eye
LOD	limit of detection
LOQ	limit of quantification
MAB	Millipore assay buffer
Mag	magnetic
MCP	monocyte chemotactic protein
MGD	meibomian gland dysfunction
MHC	major histocompatibility complex
Min STIM	minimally stimulated
MIP	macrophage inflammatory protein
MSB	LINCOplex serum matrix
N	normal
NS	non-stimulated
OOR	out of range
PBS	phosphate buffered saline
PDGF	platelet-derived growth factor
PE	phycoerythrin

PS	polystyrene
PVA	polyvinyl alcohol
PVP	polyvinylpyrrolidone
RANTES	regulated upon activation, normal T-cell expressed, and secreted
Rec	recovery
STIM	stimulated
Sub	subject
TAB	Teknova Assay Buffer
TAB-AP	Teknova Assay Buffer with anti-protease
T-cell	Thymus cell
T_C	cytotoxic T cell
T_H	helper T-cells
TNF	tumor necrosis factor
ULOQ	upper limit of quantification
VEGF	vascular endothelial growth factor
WO	washout

BACKGROUND AND SIGNIFICANCE

Ocular Surface Diseases

Dry eye is one of the most common ocular surface diseases, affecting 1.68 million men and 3.23 million women over the age of 50 in the United States. [1, 2]. Symptoms of the condition include ocular discomfort, inflammation, and decreases in visual function [3]. A common treatment for dry eye is artificial tears for temporary relief from some symptoms [4]. The main mechanisms of dry eye are tear film instability and aqueous tear deficiency. When corneal mucins do not attach to the tear film, stability is lost and more rapid tear evaporation can occur. Tear film instability is often the initial symptom in many types of dry eye [5, 6]. Aqueous tear deficiency results in tear hyperosmolarity, or an increase in solute concentration in tears.

Tear hyperosmolarity may be responsible for many of the ocular surface changes seen in aqueous tear-deficient dry eye and appears to cause inflammatory events, such as increased production of inflammatory mediators [5, 6]. Clinically, there are two main types of dry eye: aqueous tear-deficient and evaporative dry eye. Aqueous tear-deficient dry eye is a result of reduced lacrimal tear secretion, which causes hyperosmolarity and the release of inflammatory mediators. Sjögren's syndrome and non-Sjögren's aqueous deficiency are the two subtypes of aqueous tear-deficient dry eye. Sjögren's syndrome is an autoimmune disease that results in cell death and hyposecretion of tears. In non-

Sjögren's dry eye, there is damage to the lacrimal glands but the cause is not autoimmune. Evaporative dry eye occurs when there is normal secretion but too much water is lost from the exposed eye. Its causes can be intrinsic or extrinsic. Intrinsic causes include meibomian gland dysfunction and low blink rate. Meibomian gland disease, the most common cause of evaporative dry eye, causes inflammation and reduces lipid secretion onto the surface of the tear film. Extrinsic causes of evaporative dry eye include contact lens wear and vitamin A deficiency. More than one of these factors may coexist and can increase the severity of dry eye [6].

The eyelids, ocular surface, lacrimal glands, and nerves that connect them make up the lacrimal function unit (LFU). Using the more global view of the anterior eye, dry eye results from any disturbance of the LFU. In all types of dry eye, inflammation is a common theme, and the levels of inflammatory mediators, such as cytokines, are modified by the LFU [6].

Ocular Surface Inflammation

An inflammatory response involves a change in blood flow, removal of foreign antigens, and immune and phagocytic cell release into the area of tissue damage or trauma. The response can be localized or systemic. Inflammation can involve an innate or adaptive response. Innate responses provide an immediate and non-specific response to a pathogen. If this response is unsuccessful in removing the pathogen, then the adaptive response occurs with a more specific attack [7].

Dysfunction or disease of the secretory glands in the LFU can cause a change in the tear composition that then leads to production of inflammatory mediators, such as cytokines, in the tear film and on the ocular surface. The lacrimal gland, conjunctiva, and corneal epithelium can secrete cytokines [8-10]. Ocular inflammation can cause negative changes in the LFU which may lead to tear evaporation in those affected by dry eye [6]. As stated above, the inflammation has many possible causes, including contact lens wear, vitamin A deficiency, and autoimmune disease [11].

Production of Different Tear Types

Different types of tears indicate the ocular surface status. Non-stimulated (NS) tears are tears that naturally rest on the ocular surface. Stimulated (STIM) tears are tears produced by placing a cotton-tipped applicator into the nose in order to stimulate the sneeze reflex. Usually, STIM tears production is in larger volumes and at a greater rate than NS. NS contain ocular surface-derived and lacrimal gland cytokines. STIM tears contain a greater proportion of lacrimal gland cytokines relative to NS because these tears are diluted more by lacrimal gland secretion.

Cytokines

Cytokines, which are present in all tear types, are cell-signaling proteins released by white blood cells or other cells in response to a stimulus, such as inflammation or tissue injury [7] . Types of cytokines include interleukins, chemokines, tumor necrosis factors, and interferons. Cytokine is the collective name for these types of cytokines. The

main producers of cytokines are helper T-cells, macrophages, and dendritic cells [7]. When cytokines bind to receptors on the cornea and conjunctiva, signal transduction occurs followed by gene activation. Changes in gene expression lead to various biological effects [12]. The many biological functions mediated by cytokines include regulation of cellular activity, development of immune responses, and initiation of inflammatory responses [7].

Cytokines have various characteristics that allow cell activity to be controlled. They exhibit pleiotropy, which means a single cytokine can produce several effects, and redundancy, which means more than one type of cytokine can cause the same effect. Cytokines can also act synergistically, where the combined activity of more than one cytokine can create a greater effect than if each acted individually, or antagonistically [7].

Immune responses by lymphoid cells, inflammatory cells, and hematopoietic cells are cytokine-modulated. Cytokines can induce action of immune effector cells or create an immune effect themselves. They usually have an autocrine effect, acting on the same cell that produced it, or paracrine effect, acting on nearby cells. However, they can sometimes have an endocrine effect by acting on other areas of the body via the bloodstream. Binding by cytokines is generally nonspecific. Specificity of the immune response is maintained because some cytokine receptors may only be expressed after an antigen has interacted with that cell or after a target cell interacts with a cytokine-producing cell [7].

T-cell Release of Cytokines

Lymphocytes are mononuclear leukocytes, or white blood cells, that mediate humoral immunity by B-cells, or cell-mediated immunity by T-cells. T-cells mature in the thymus and express a unique receptor on its membrane. Only antigens bound to an antigen-major histocompatibility complex (antigen-MHC) on an antigen-presenting cell (APC) interact with T-cell receptors. The two types of MHCs are class I and class II MHC molecules. Expression of class I MHC molecules occurs on nearly all nucleated, vertebrate cells, whereas, class II MHC molecules are restricted to only a few cell types. When this interaction between T-cells and antigen-MHC complex occurs, the T-cell differentiates into effector and memory cells. Effector cells carry out cell-mediated responses. Memory cells express the same antibody as the parent T-cell and have a longer lifespan than effector cells [7].

Two types of T-cells are helper T-cells (T_H) and cytotoxic T-cells (T_C). T_H cells usually display CD4 glycoproteins on its membrane. Helper T-cell production of cytokines has often been classified into either T_H1 or T_H2 patterns [7, 13]. Each production pattern produces a different cytokine response. T_H1 cells promote a cell-mediated response with the production of interferon-γ (IFN-γ), interleukin 2 (IL-2), and tumor necrosis factor β (TNF-β). T_H2 cells can cause a humoral response with the production of IL-4, IL-5, IL-6, and IL-10 [14]. Past research has concluded that $T_H1$cells are associated with pro-inflammatory responses and T_H 2 with an anti-inflammatory response [15]. Past researchers believed that the cytokines associated with one pathway would inhibit the oth-

er. However, recent research shows the cytokine profile of T_H cells to be a mixture of T_H1 and T_H2 responses and not solely one or the other [16].

In addition to T_H1 and T_H2 patterns, cytokine production may be involved in another pattern, T_H17, which involves IL-17 and causes chronic inflammation. IL-17 may be associated with inflammatory diseases such as dry eye [17]. Dysfunction in regulation of T-cells is associated with the increase in IL-17. Blocking IL-17 has reduced the severity of some symptoms of dry eye disease [18].

Many types of cytokines are in the body and could be present in the tears of dry eye patients. The amount and type of cytokines in the tear film may indicate inflammation. The cytokine profile of individuals with ocular surface diseases tends to differ from that of individuals without such disease. Diagnosis and treatment of dry eye dysfunctions could be improved if an accurate cytokine profile of the disease types was known [19]. Identifying the cytokine profile of different types of dry eye through assays could allow clinicians and researchers to develop targeted treatments. Previous studies of tear cytokines only tested a limited number of inflammatory markers, such as IL-6, IL-8 and tumor necrosis factor α (TNF-α), instead of a broader approach allowing a fuller understanding of all cytokines that may be involved. There are various types of assays used to determine the type and amount of cytokines in tears.

Assay Types for Cytokine Detection

Sandwich enzyme-linked immunosorbent assay (ELISA) runs on a microtiter plate with primary antibody already attached to individual wells. A sample containing the

antigen of interest added to each well attaches to the primary antibody at a specific epitope. Next, a biotinylated second antibody added to the wells attaches to the antigen at a different epitope. Then, there is addition of an enzyme-linked streptavidin-peroxydase, which has a high affinity for biotin. Finally, the added substrate reacts with streptavidin-peroxydase and creates a colored product whose intensity will determine the concentration of the antigen. Incubation is next to allow binding. Washing occurs after each step to remove unbound material [7, 20]. ELISA is widely known as the gold standard for antigen detection [21, 22]. However, ELISA only allows the assay of a single antigen in a given sample, whereas, multiplexed bead-based assays (BBAs) allow immunoassay of multiple antigens in a given sample. Sandwich ELISA has the primary antibody attached to the wells and BBAs have the primary antibody attached to suspended beads.

Multiplexed BBAs use microspheres colored with red and infrared dyes. There are 10 specific concentrations of each dye to create 100 different unique combinations of bead colors. Each of these beads has a distinct bead identification number that emits a unique fluorescence of the red and infrared dyes. The microspheres are coated with specific capture antibody. When a sample is added to the solution, the antigen, or cytokine in this study, is captured by specific microspheres. Detection antibodies specific to the cytokines being analyzed are added to create a sandwich and then a fluorescent tag, streptavidin-phycoerythrin (PE), is affixed to all antibody sandwiches. Each sample of microspheres is analyzed using lasers that excite the dyes on the bead surface to determine the type and amount of cytokine. A red laser determines the classification of the bead color

and a green laser determines the amount. Unlike ELISA, multiple microspheres which are specific for many different cytokines can be analyzed in a single well [23, 24].

All BBAs in this study contained dilutions of standards, which contained a known concentration of cytokines, for comparison with tear samples. Standards were diluted to various concentrations to determine the sensitivity of the assays at different levels. The standard curve, which graphed the standard concentrations (x-axis) against the fluorescence reading (y-axis), was created for each BBA. The standard curve was analyzed to see if the tear sample cytokine concentrations corresponded to the known standard cytokine concentration [25].

Polystyrene versus Magnetic Beads

Polystyrene beads have been the main type of beads manufactured and used in BBAs. The magnetic beads, which are polystyrene beads coated with magnetic (iron) particles, have become the primary bead choice due to changes in industry manufacturing [23]. Therefore, determination of optimal magnetic BBA conditions and techniques is necessary. Polystyrene and magnetic BBA procedures are similar but with some notable differences. First, polystyrene beads are 5.6 microns in diameter and magnetic beads are 6.5 microns in diameter (Figure 1). In addition, magnetic beads have a lower density than polystyrene beads in BBA kits. Some companies state that the two bead types should yield identical results for a given assay, but this has not been the case in this lab.

Figure 1. Polystyrene and Magnetic Bead Diameters.

Technique-Based Differences: Assay Plates and Washing Procedures

Washing during all immunoassays removes unbound substances to allow for a better adherence of cytokines and antibodies to beads. Wash steps for polystyrene and magnetic beads can both use the manual or automatic wash system. Filter plates can be used in both systems with both bead types and plastic can only be used in the automatic system with magnetic beads.

The manual wash/vacuum system used was the MultiScreen® Resist Vacuum Manifold (Millipore Corp., Billerica, MA). It has a vacuum pump and pressure gauge to set a desired pressure for vacuuming. After the operator placed a filter plate on the manifold, a foot pedal controls the amount and duration of suction from below. The operator has to hold the four corners of the filter plate during vacuuming to ensure even distribution of suctioning. Manual vacuuming takes longer than and may not be as efficient as the automatic wash system because of human error.

Vacuum filtration from below or aspiration from above occurs during automatic wash steps to separate antibodies and cytokines from buffer solutions. The Tecan HydroFlex™ microplate washer (Tecan Group Ltd., Mannedorf, Switzerland) is the automated washer used for vacuum and aspiration. It has an interchangeable vacuum manifold and magnetic plate carrier. For both wash methods, assay buffer is dispensed into each well from above. With vacuum filtration, assay buffer is suctioned from wells from below. Aspiration wash steps require a plastic plate and involve aspiration from above of buffer. The magnetic plate carrier has 96 small magnets that go underneath the plastic plate and cause adherence of beads to the middle of all wells as fluid is aspirated.

Vacuum filtration usually results in residual volume that may decrease assay efficiency. Using aspiration steps with the plastic plate for magnetic BBAs nearly eliminates this problem. With aspiration of magnetic beads, well integrity should remain intact and improve consistency from one well to the next. Therefore, plastic plates using aspiration would be expected to be superior to vacuum filtration, but previous results described below have shown otherwise.

Replicate tear samples run on past BBAs have shown different results when using the different wash methods. Replicates of a given sample are expected to have 0% intra-assay coefficients of variation because they are from the same pool. However, past studies did not produce such ideal results [26]. From observations of past BBAs in this lab, tear sample intra-assay coefficients of variation in descending order were manual vacuum, automatic vacuum with filter plate, and automatic with plastic plate. Based on

these results, the automatic wash system using the plastic plate and magnetic beads would be expected to give optimal results but this may not be the case.

Assay Interference

Interference can occur in assays from interaction by the following tear components: lysozyme, lactoferrin, lipocalin, surfactants, and other endogenous proteins. Lysozyme and lactoferrin have shown some interference in tear BBAs [27]. Tear lipocalin may also contribute to interference caused by lysozyme and lactoferrin because these proteins have been reported to bind to each other when secreted from the lacrimal gland [28]. Lactoferrin has iron-binding properties that may interact with the iron particles on the magnetic bead surface during assay binding steps [29]. This interaction between tear lactoferrin and the magnetic beads may cause a false increase or decrease in binding. Boonstra et al [30] reported interference by tear surfactants in direct antigen-binding ELISA. Surfactants present in tears may cause interference by inhibiting the binding of proteins to solid surfaces, such as beads [30].

Endogenous proteins in serum samples have been found to adversely affect results in BBAs by causing interference [31]. There is a group of endogenous proteins, called heterophilic antibodies, with interspecies specificity that can interfere in immune-based assays [32]. Heterophilic antibodies are present in humans and can bind to either animal or human antibodies. If these antibodies are present in tear samples, then they can bind animal antibodies present in BBA buffers, which contain animal serum. In BBAs, heterophilic antibodies can create bridges between capture and detection antibodies. These

bridges can falsely decrease cytokine detection by blocking capture antibody binding sites or increase cytokine detection. This interspecies binding can lead to false quantification of cytokines in BBAs [33, 34]. In some studies, high cytokine levels caused by endogenous agents or antibodies may have been incorrectly identified as outliers instead of being attributed to interference [34]. Some of these interfering agents may contribute to variability in assay results between research groups.

Previous Interference Studies

ELISA and polystyrene BBA studies. Earlier bead-based and ELISA-based assays of tear IL-8 and interferon-inducible protein 10 (IP-10) demonstrated that some tear components might be interfering with the BBAs [35, 36]. Sack also reported interference in multiplexed tear assays, using an antibody array membrane. He concluded that IL-8 levels were artificially elevated in the multiplex assays [37].

Tran and Guyette found interference involving IP-10 and IL-8 in a study of matched samples run on an ELISA and a polystyrene BBA [35]. Different buffers were used for each assay type. Tear samples were diluted in Millipore assay buffer (MAB) for the BBA and diluted in Diaclone standard diluent buffer (DAB) for the ELISA. They quantified IP-10 at much higher levels in the polystyrene BBA than the ELISA did. On the other hand, IL-8 concentration was much greater in the ELISA than the polystyrene BBA. These differences were possibly due to interference because polystyrene BBA concentrations would be expected to be similar to ELISA concentrations when matched samples are used.

In the matched ELISA and polystyrene BBA results, there was only a weak correlation between the ELISA and polystyrene BBA of IP-10 for NS tears, while there was a strong correlation between the two assay types for STIM tears. On the other hand, IL-8 showed positive correlation between the results using ELISA and polystyrene BBAs for both NS and STIM tears. Interference was indicated with IP-10 in the polystyrene BBA because there was a weak correlation between the two assay types for NS tears. With IL-8, interference was indicated in the polystyrene BBA because it had 2.7 times lower concentrations than the ELISA [35].

Polystyrene and magnetic (filter plate) BBAs. Another statistical measure, covariance, has suggested different levels of interference in previous BBAs. Cytokine covariance is a measure of the extent to which two cytokine concentrations change together. Cytokines tend to show group patterns of covariance because they often work agonistically or antagonistically. In a 27-Plex polystyrene BBA conducted by Kehinde et al [38], multiple linear regression showed covariance between IL-8 and one other cytokine, granulocyte colony-stimulating factor (G-CSF) (Table 1). This means that the concentration of IL-8 could be predicted by the concentration of only one other cytokine in the polystyrene BBA. This is surprising because given the nature of cytokine redundancy more covariance would be expected.

Table 1

Covariance of Tear IL-8 with Other Cytokines in 27-Plex Polystyrene BBA

Independent Variable	P value
IL-1β	0.501
IL-1ra	0.619
IL-2	0.149
IL-4	0.083
IL-5	0.892
IL-6	0.261
IL-7	0.679
IL-9	0.114
IL-10	0.466
IL-12p70	0.956
IL-13	0.068
IL-15	0.252
IL-17	0.862
Eotaxin	0.745
FGF basic	0.164
G-CSF	0.035
GM-CSF	0.768
IFN-γ	0.999
IP-10	0.188
MCP-1	0.430
MIP-1α	0.517
MIP-1β	0.765
PDGF-BB	0.335
RANTES	0.702
TNF-α	0.724
VEGF	0.630

BBA = bead-based assay. P-value <0.05 indicates significant covariance with IL-8 (dependent variable). Data from Kehinde, et al [38]. Refer to list of abbreviations (Page xv) for cytokine identification.

In contrast, multiple linear regression showed covariance between IL-8 and five other cytokines in a filter plate-based magnetic BBA run in conjunction with Bausch and Lomb (no conflict of interest) (Table 2) [39]. In this assay, concentrations of the following cytokines have the ability to predict the concentration of IL-8: IL-6, granulocyte macrophage colony-stimulating factor (GM-CSF), IFN-γ, monocyte chemotactic protein-1 (MCP-1), and macrophage inflammatory protein-1β (MIP-1β). The covariance within the magnetic BBA is therefore much stronger than in the polystyrene BBA. There is a positive relationship between IL-8 and two (IL-6 and IFN-γ) of the five covarying cytokines. IL-6 is expected to covary with IL-8 because they are both typically produced after tissue injury or infection. IL-6, which can be pro-(T_H1) or anti-inflammatory (T_H2), is often induced by IFN-γ, which is pro-inflammatory [40]. While this may be indicative of true covariance among cytokines, it is possible that it indicates greater interference in the magnetic BBA.

Table 2

Covariance of Tear IL-8 with Other Cytokines in 27-Plex Magnetic BBA

Independent Variable	P value
IL-1β	0.373
IL-1ra	0.851
IL-2	0.154
IL-4	0.278
IL-5	0.953
IL-6	0.006
IL-7	0.079
IL-9	0.746
IL-10	0.398
IL-12p70	0.339
IL-13	0.516
IL-15	0.524
IL-17	0.902
Eotaxin	0.188
FGF basic	0.068
G-CSF	0.203
GM-CSF	0.023
IFN-γ	0.018
IP-10	0.077
MCP-1	0.035
MIP-1α	0.054
MIP-1β	<0.001
PDGF-BB	0.666
RANTES	0.201
TNF-α	0.055
VEGF	0.051

BBA = bead-based assay. P-value <0.05 indicates significant covariance with IL-8 (dependent variable). Data from Merchea, et al [39]. Refer to list of abbreviations (Page xv) for cytokine identification.

In addition to greater covariance with this Bausch and Lomb magnetic BBA, tear cytokine levels were generally higher than for the polystyrene BBA [38] (Table 3). The average levels of 25 out of 27 cytokines were significantly higher for this magnetic BBA than the polystyrene BBA. IL-8, IP-10, and IFN-γ, which have previously shown interference, were among those showing greater magnetic than polystyrene BBA values.

Table 3

27-Plex Polystyrene and Magnetic BBA Comparison (Non-stimulated Tear Samples)

	Cytokine	Number of Samples	Missing Data	Mean	SE
Magnetic (filter plate)	IL-8	72	0	901.14	26.1
	IFN-γ	72	4	2,835.69	74.52
	IP-10	72	0	10,303.99	1,354.74
Polystyrene	IL-8	72	0	554.56	27.77
	IFN-γ	72	3	1,264.50	54.65
	IP-10	72	0	3,735.06	171.21

Tear concentrations in pg/mL. BBA = bead-based assay. SE = standard error. Eight normal subjects. Data from Kehinde, et al [38].

Only TNF-α and GM-CSF had polystyrene BBA values that were greater than magnetic BBA values. It is possible that an interfering agent is producing higher cytokine levels in the magnetic BBAs. With identical standards for both plates, similar results would be expected.

Polystyrene and magnetic (plastic plate) BBAs. Several polystyrene BBAs conducted in other labs show levels of tear cytokines varied within a given tear sample. A plastic plate-based magnetic BBA in the Bausch and Lomb study showed cytokines as either all low or all high within a given subject. In contrast to polystyrene BBAs, if one cytokine was elevated, then all cytokines were high in these plastic plate-based magnetic BBAs.

Interference has been a major problem on magnetic BBAs run on plastic plates [27]. Cytokine covariance of a polystyrene BBA [38] was compared to a plastic plate-based magnetic BBA [27] (Table 4). There was no cytokine covariance in the polystyrene BBA. On the other hand, many cytokines covaried in the magnetic BBA. For the magnetic BBA, positive covariance means the cytokine levels change in the same direction with both increasing or both decreasing together. For example, as IP-10 levels increase, vascular endothelial growth factor (VEGF) and platelet-derived growth factor (PDGF-bb) levels would be expected to increase. Negative covariance means the cytokine levels change in opposite directions. In this case, if IP-10 levels increases, then IL-12p70 would decrease. This considerably greater amount of covariance with the magnetic BBA may indicate true covariance or greater interference than the polystyrene BBA.

Table 4

Covariance of Tear Cytokines on Polystyrene and Magnetic (plastic plate) BBAs

	Covarying Cytokines		
	Polystyrene	Magnetic	
		Positive	Negative
IL-1β	-	IL-4, MIP-1β, TNF-α, PDGF-bb	-
IL-1ra	-	PDGF-bb, IL-6	IL-10, FGF basic, MCP-1
IL-2	-	RANTES	-
IL-4	-	-	-
IL-5	-	-	-
IL-6	-	IL-1ra, FGF basic	-
IL-7	-	IL-12p70	-
IL-8	-	G-CSF	-
IL-9	-	IL-13	-
IL-10	-	MIP-1β, PDGF-bb	IL-1ra, MCP-1
IL-12p70	-	IL-7, VEGF	IP-10, MIP-1α
IL-13	-	IL-5, IL-9, IL-15, G-CSF	-
IL-15	-	IL-13, VEGF	-
IL-17	-	MIP-1α	TNF-α
Eotaxin	-	-	RANTES
FGF basic	-	IL-6, MIP-1α	IL-1ra
G-CSF	-	IL-8, IL-13	-
GM-CSF	-	IFN-γ, VEGF	-
IFN-γ	-	GM-CSF	-
IP-10	-	VEGF, PDGF-bb	IL-12p70
MCP-1	-	IL-5, MIP-1β, PDGF-bb	IL-1ra, IL-10
MIP-1α	-	IL-17, FGF basic	IL-12p70
MIP-1β	-	IL-1β, IL-10, MCP-1	PDGF-bb
PDGF-bb	-	IL-1β, IL-1ra, IL-10, IP-10, MCP-1	MIP-1β, VEGF
RANTES	-	IL-2	Eotaxin
TNF-α	-	IL-1β	IL-17
VEGF	-	IL-12p70, IL-15, GM-CSF, IP-10	PDGF-bb

Data from Fullard, et al and Kehinde, et al [27, 38]. BBA = bead-based assay. Refer to list of abbreviations (Page xv) for cytokine identification.

Figure 2 shows data from a 27-Plex filter plate-based magnetic BBA [27], polystyrene BBA [38], and plastic plate-based magnetic BBA [27]. Within each assay, a subject's concentration for each of the 27 cytokines was ranked. Then, the means of these rankings were calculated for each subject. Subject numbers were assigned based on these mean rankings from low to high. A slope closer to zero indicates there is less covariance and a slope near one indicates there is more covariance. Mean cytokine rankings were similar for the polystyrene BBA (red line) and the filter plate-based magnetic BBA (blue line). The slopes for these two BBAs were both closer to zero. For the plastic plate-based magnetic BBA (black line), the slope was closer to one than the other two BBAs, which indicates it has a higher degree of covariance than the other two BBAs. The lowest ranked subject had the lowest mean cytokine rank and the highest ranked subject had the highest mean cytokine rank. In other words, subject 1 has the lowest cytokine levels for most cytokines and Subject 9 has the highest cytokine levels for most cytokines. Showing this trend of most cytokines being low or most being high within a given subject is contrary to the expected variation of cytokine levels within a given subject.

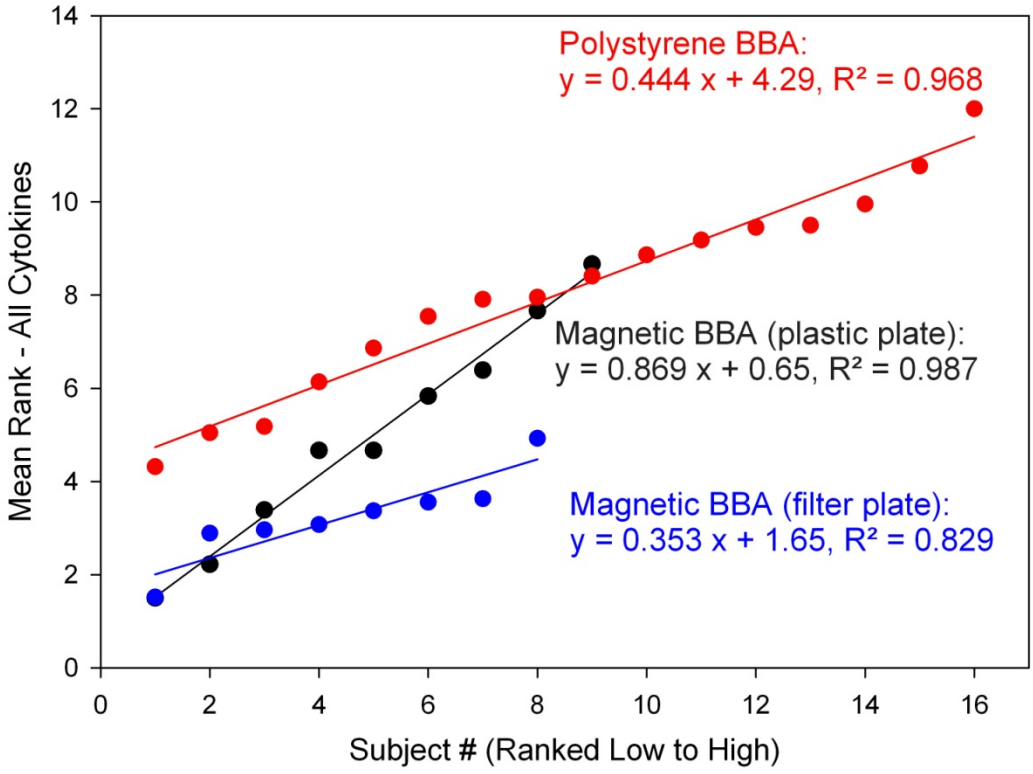

Figure 2. Overall Mean Ranking by Subject for Various BBAs. BBA = bead-based assay. Data from Fullard, et al and Kehinde, et al [27, 38]. Steeper slope indicates less cytokine variation within each subject. Least cytokine variation with magnetic BBA (plastic plate).

Another polystyrene BBA run by Kehinde et al had coefficients of variation (CVs) between 8% and 16% [38]. CVs for a plastic plate-based magnetic BBA [27] were between 7% and 8%, which, on average, were much lower than the equivalent polystyrene BBA CV. Low CVs may have occurred with plastic plate-based magnetic BBAs because of a more efficient washing system (aspiration). This indicates magnetic BBAs may have an advantage over polystyrene BBAs, although, the lower coefficients of variation could be due to uniform interference [26].

Assay interference in other labs. Other researchers have not found magnetic BBA interference like what has occurred in this lab. However, these BBAs were based on other body fluids. Assays of lung lavages, which contain similar inflammatory markers, did not show the systematic interference that was in the early tear magnetic BBAs run in this lab [41, 42]. However, matrix effects, or the combined effect of all factors other than cytokines within the tear sample that can affect appropriate binding of cytokine to beads, have been reported in multiplex microplate array assays of tear cytokines. These matrix effects would have decreased assay reliability and would have been more noticeable without using a buffer before the assay was run that could block matrix effects [43]. Matrix effects may also be present in tear magnetic BBAs and create similar issues with assay reliability.

Influence of Assay Buffer on Interference

Buffers containing proteins and surfactant are commonly used to counteract interference. Many of the buffers used as sample diluents contain animal sera and proprietary components. Bovine serum albumin (BSA), fetal bovine serum (FBS), and Tween 20 are examples of common buffers used to block interference [44]. A study investigating the effectiveness of various buffers showed 10% FBS to be the best by blocking interference in nearly half of the samples tested. Non-immune mouse serum was second to 10% FBS in blocking effectiveness in that study. 10% FBS has also been shown to reduce binding by heterophilic antibodies [45].

SPECIFIC AIMS

1. Define an optimal magnetic BBA for NS tears that includes as many cytokines as possible that give equivalent results to polystyrene BBAs.
2. Determine the origin of differences between polystyrene and magnetic BBAs of tears using the Luminex® 200™ system (Luminex Corp., Austin, TX):
   i. Technique-based (wash methods; plate types)
   ii. Tear interference with magnetic beads.
3. Test magnetic BBA modifications and buffers using different tear sample types to produce magnetic BBA results equivalent to those of polystyrene BBAs.

Null Hypothesis: Optimal technique modifications and assay buffers do not produce equivalent results for polystyrene and magnetic bead-based Luminex assays of tear cytokines.

Research Hypothesis: Optimal technique modifications and assay buffers produce equivalent results for polystyrene and magnetic bead-based Luminex assays of tear cytokines.

Rationale

Bio-Rad® (Hercules, CA) magnetic BBAs have replaced their earlier polystyrene BBAs. Some covariance is expected among cytokines because of their properties of redundancy (several cytokines performing similar functions at a particular tissue site) and pleiotropism (the property of cytokines that their function differs at different inflammatory sites or under different inflammatory conditions). The initial magnetic BBAs run with MAB showed almost universal positive covariance among cytokines. This is strong evidence for systematic assay interference. If this can be successfully removed, the true covariance among cytokines may become more evident. Therefore, using appropriate buffers and making modifications to assay techniques to reduce interference could produce magnetic BBA results approximately equivalent to polystyrene BBAs.

EXPERIMENTAL DESIGN AND METHODS

Overview

Individuals of any age with or without dry eye were recruited for study participation (Table 5). NS, washout (WO), minimally stimulated (Min STIM) and STIM tears were collected for use in multiple, developmental BBAs. LINCOPlex® (Millipore Corp. Billerica, MA) and single and multiplexed Bio-Plex® assay kits (Bio-Rad Laboratories, Inc., Hercules, CA) were used. These assays involved various techniques, equipment, and buffers. All procedures were approved by the University of Alabama at Birmingham Institutional Review Board for Human Use (IRB).

Table 5

Subject Numbers and Dry Eye Disease Descriptions for All Polystyrene and Magnetic BBAs in this Thesis

Subject Number	Normal/Dry	Description	Basis
1	N	Normal	Asymptomatic
2	N	Normal	Asymptomatic
3	N	Normal	Asymptomatic
4	N	Normal	Asymptomatic
5	N	Normal	Asymptomatic
6	N	Normal	Asymptomatic
7	N	Normal	Asymptomatic
8	N	Normal	No clinical signs*
9	N	Normal	Asymptomatic
10	N	Normal	No clinical signs*
11	N	Normal	Asymptomatic
12	N	Normal	Asymptomatic
13	N	Normal	Asymptomatic
14	LN	Lupus non dry eye	No clinical signs*
15	EDE	Evaporative	Confirmed MGD*
16	EDE	Evaporative	Clinical Signs*
17	EDE	Evaporative	Clinical Signs*
18	EDE	Evaporative	Clinical Signs*
19	D	Dry Eye	Symptomatic
20	D	Dry Eye	Symptomatic
21	AE	ADDE and EDE	Clinical Signs*
22	AE	ADDE and EDE	Clinical Signs*
23	ADDE	Punctal plugs	Symptomatic
24	ADDE	Aqueous-deficient	Clinical Signs*

*Clinically evaluated. EDE = evaporative dry eye. AE = aqueous-deficient and evaporative dry eye. ADDE = aqueous-deficient dry eye.

Based on the preceding background information, literature reports, manufacturer recommendations, and previous assay results in this lab, the following buffers were compared to determine which was most effective in blocking interfering agents in tears: Bio-Plex assay buffer (BAB), Bio-Plex human serum standard diluent (BSD), custom Brookwood Biomedical serum buffer (BBS), Diaclone standard diluent buffer (DAB), Millipore assay buffer (MAB), Millipore serum buffer (MSB), and Teknova assay buffer (TAB) (Table 6). Many of these buffers were chosen because of their serum composition or recommended by manufacturers. Some of the proprietary buffers were believed to contain animal serum, such as fetal bovine serum.

Table 6

Composition of Bead-based Assay Buffers

Buffer	Composition
Bio-Plex® assay buffer (BAB)	Proprietary
Bio-Plex® human serum standard diluent (BSD)	Proprietary
Brookwood Biomedical serum buffer (BBS) (Custom)	5% normal mouse serum, borate buffered saline, 1% bovine serum albumin (BSA), 1% gelatin, 0.1% Tween 20, pH 8.4
Diaclone™ standard diluent buffer (DAB)	Proprietary serum; also used in IL-8 and IP-10 ELISAs
Millipore™ assay buffer (MAB)	Proprietary
Millipore™ serum matrix (MSB)	Proprietary
Teknova® assay buffer (TAB)	1% BSA, 0.1% sodium azide, 0.05% Tween 20, pH 7.4, phosphate buffered saline (PBS)
Teknova® assay buffer with anti-protease (TAB-AP)	TAB with SigmaFast™ Protease Inhibitor Cocktail Tablet, EDTA Free

Tear Collection and Storage

All tear samples were collected from normal and dry eye subjects. NS tears were collected using 10 µL polished glass micropipettes (Drummond Scientific Company, Broomall, PA). The subjects themselves or an investigator collected the tears by placing the microcapillary tube into the tear meniscus at the lower lid margin. Minimal contact was made with the ocular surface. Care was used to reduce the likelihood of reflex tearing.

WO tears were obtained by adding 10 µL of sterile saline solution to the lower fornix of the collecting eye and the contra-lateral eye. Saline was added to the contra-lateral eye before the collecting eye in order to prevent that eye from drying out. Next, the subject closed their collecting eye for 1 minute to minimize drainage of saline solution. The subject collected WO tears in 5-minute increments or until 6.5 µL of tears was collected. Samples were immediately stored and collection continued until the tear flow rate began to slow to less than 6.5 µL in a 5-minute period. Then, another 10 µL of sterile saline solution was added to the lower fornix and the 5-minute increment collections continued until the tear flow rate slowed again.

Min STIM tears were also collected using 10 µL polished glass micropipettes. These tear collections were similar to NS tear collections, except contact with the ocular surface and the flow rate were not monitored.

STIM tears were collected by gently inserting a cotton-tipped applicator into the nasal passage to generate a sneeze reflex. The tip was moved around until the eyes began

to well-up. The tears were collected throughout this process using 20 µL polished glass micropipettes.

All tear samples were diluted one in 10 with buffer. For the final 27-Plex polystyrene and magnetic BBA comparisons, 9 µL of buffer was pre-added to the tubes to reduce evaporation and breakdown of cytokines at the beginning of the storage process. Samples were stored in a 0.2 mL, 0.5 mL or 2.0 mL tube and placed in a -80°C freezer until the day of the assay. Various assays were performed on tear samples.

Bead-based Assay Procedure

Polystyrene BBAs used filter plates while magnetic BBAs used either filter or plastic plates. The wash steps for both bead types used the manual or automatic wash apparatus. Standard samples were included on all assays.

Assay procedure began with preparation of multiplex bead working solution from polystyrene (25x concentration) or magnetic beads (10x concentration). For polystyrene beads, one-part beads and 24 parts assay buffer comprised the working solution. For 1-Plex, 11-Plex, and 27-Plex magnetic beads, one-part beads and nine parts assay buffer. The 3-Plex magnetic BBA had one part of each cytokine bead stock and seven parts assay buffer. Each well of the 96-well plate was wet with 100 µL of BAB and vacuumed or aspirated using the MultiScreen Resist Vacuum Manifold or the Tecan HydroFlex microplate washer. For each well, 50 µL of the bead working solution was added. Then, the plate was washed and vacuumed twice. Residual fluid was wiped from under the filter plates after each wash step for all assays that used filter plates. Each well had 50 µL of

standards and samples added. The entire plate was covered with sealing tape, wrapped in aluminum foil, and secured with tape in preparation for incubation. Shaker speed began at the maximum setting of 1,100 rpm for 30 seconds. Then, the speed was reduced to 300 rpm for 60 minutes at room temperature.

For polystyrene, 1-Plex magnetic, 11-Plex, and 27-Plex magnetic BBAs, detection antibody solution was prepared with one-part detection antibody and nine parts detection antibody diluent. Detection antibody solution for the 3-Plex magnetic BBA was comprised of one part of each cytokine detection antibody and seven parts detection antibody diluent. After the 60-minute incubation, the plate was washed and vacuumed 3 times. Each well had 25 µL of the detection antibody working solution added. The entire plate was covered with sealing tape, wrapped in aluminum foil and secured with tape in preparation for incubation. Shaker speed again began at the maximum setting of 1,100 rpm for 30 seconds, followed by a reduction to 300 rpm for 30 minutes at room temperature.

The fluorescent tag, streptavidin-PE, was prepared in the same manner for all assay types. One part streptavidin-PE and 99 parts BAB. After detection antibody incubation, the plate was washed and vacuumed 3 times. Each well had 50 µL of the streptavidin-PE solution was added. The plate was prepared in a similar manner as above for incubation. Shaker speed began at 1,100 rpm for 30 seconds, followed by a reduction to 300 rpm for only 10 minutes at room temperature. The plate was washed and vacuumed after the incubation period. The final step before analysis was to re-suspend the beads in 125 µL of BAB. Next, the plate was placed on the Luminex instrument for analysis using

Bio-Plex Manager™ 6.0 software. The use of the Luminex instrument and Bio-Rad reagents have been proven to be optimal in tear BBAs [46].

Assay Descriptions

Various assay kits testing a different number, or plex, of cytokines were used in preliminary studies to begin to determine optimal techniques and buffers (Figure 3). In the past, MAB or BAB was used in this lab as the standard storage buffer for tear samples. These two buffers along with many others were tested in various assays to determine the optimal buffer (Table 6). Different wash systems were also compared.

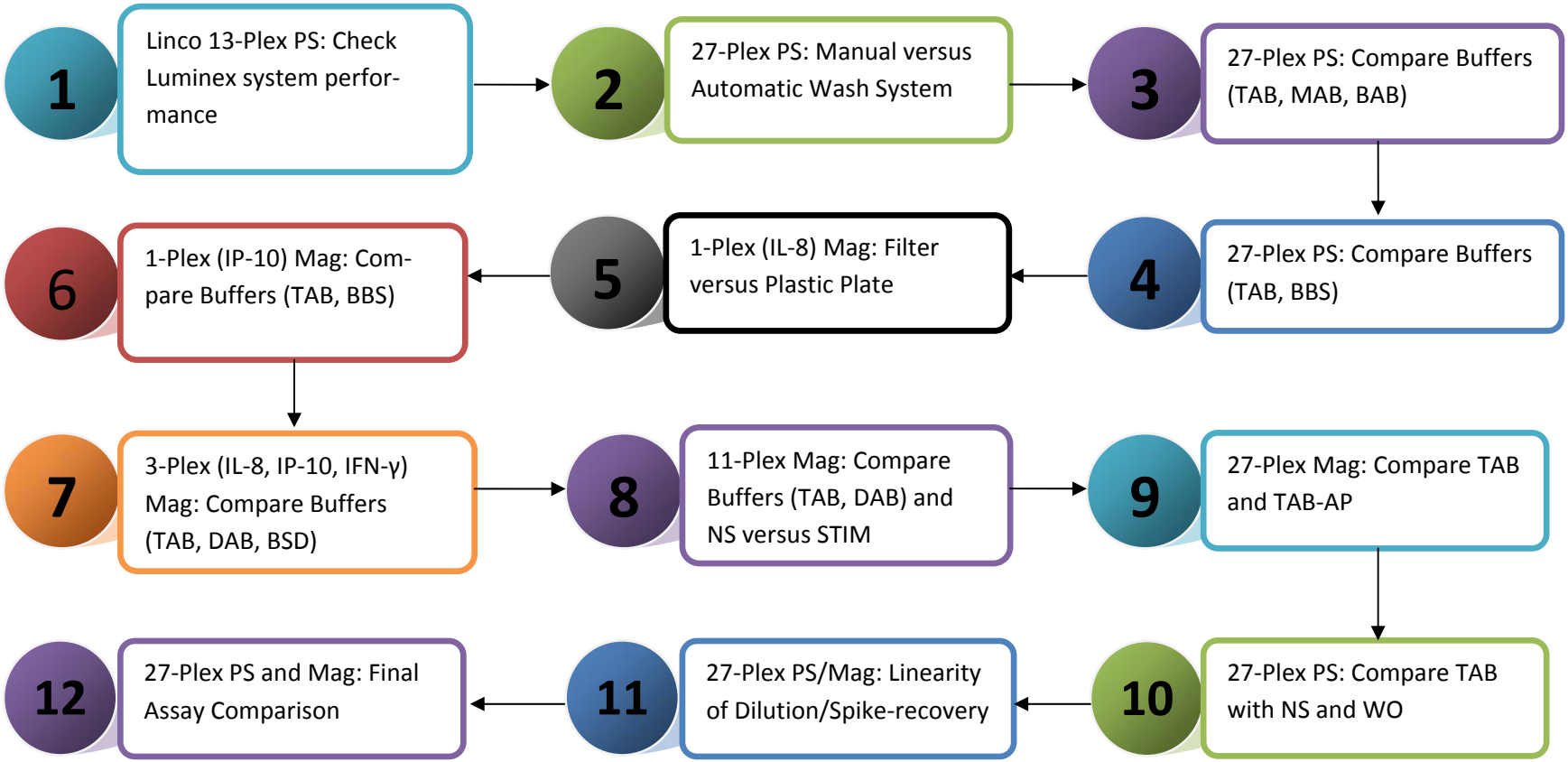

Figure 3. Flowchart of Assay Development: Polystyrene (PS) and Magnetic (Mag) BBAs.

System Performance (Flowchart #1)

System performance of the Luminex instrument, technology used to analyze cytokines, chemokines, and growth factors, were checked using the High Sensitivity LINCOplex kit (13-Plex). The buffers MAB and MSB (see Table 6) were compared for this assay. The STIM tears of seven subjects (normal: 1, 2, 5; dry: 15, 17, 21, and 23), were collected and stored in each of the buffers at -80°C. Quadruplicate wells were assayed for each subject. The samples were assayed on a filter plate and the automatic plate washer (Tecan HydroFlex microplate washer) performed the vacuum and multi-wash steps.

Manual and Automatic Wash Systems (Flowchart #2)

The manual and automatic wash systems were compared using the Bio-Rad 27-Plex polystyrene kit. Min STIM tears of Subject 21 (dry eye) were collected and stored in TAB (see Table 6) at -80°C. Two different filter plates were used for the manual and automatic wash systems with only one column assayed per plate. Triplicate wells were assayed for the subject on each plate. The manual system involved manual application of wash buffer and use of the manual vacuum apparatus. Care was taken to ensure equal and accurate application of wash buffer and that vacuuming was equal across all wells. The automatic system used the automatic plate washer to perform single wash and vacuum steps.

Buffer Comparisons (Flowchart #3 and #4)

TAB, BAB, and MAB (#3). The following buffers were evaluated using the Bio-Rad 27-Plex polystyrene kit: TAB, MAB, and BAB (see Table 6). STIM tear samples

from Subject 15 (dry eye) were collected and stored in each of the three buffers at -80°C. Three-quarters of an assay plate was used. Quadruplicate wells of pooled STIM tears were assayed for the subject in each buffer. The assay used a filter plate and multi-wash steps on the automatic system.

TAB and BBS (#4). The purpose of the next assay was to compare the buffers, TAB and BBS (see Table 6), by looking at sensitivity and linearity of dilution as measures of how well a buffer reduces interference. NS and STIM tears of two normal subjects (1 and 2) were collected and stored in each of the buffers at -80°C. For TAB, NS tears were assayed in duplicate wells for each subject and STIM tears pooled for Subject 1 in triplicate wells. For BBS, NS tears were assayed in duplicate for one subject. The Bio-Rad 27-Plex polystyrene kit was used on a filter plate with single automatic wash steps.

Filter Plate and Plastic Plate (Flowchart #5)

Magnetic assays can be run on filter plates, which allow vacuuming of wash buffer, or plastic plates, which use aspiration from above to remove wash buffer. An IL-8 single-plex Bio-Rad magnetic kit was used to evaluate the NS tears of six subjects (normal: 1, 2, and 5; dry: 20, 21, and 22) and the STIM tears of two subjects (normal: 1 and 2). Parallel samples were collected and stored in TAB (see Table 6) at -80°C. Two different plates were used with only half of a filter plate and half of a plastic plate being assayed. For each plate, duplicate wells of NS and STIM were assayed for two subjects

(normal: 1 and 2) and single wells of NS for the other four subjects (normal: 5; dry: 20, 21, and 22). Single wash steps were performed on the automatic system.

If the magnetic BBA produced interference, then we would have seen a departure from ELISA data. If filter and plastic plate results were similar, then plastic would have been the optimal plate because it is automated. If plate results were different, then filter plates would be more favorable for future assays because filter plates have previously been shown to give acceptable results similar to ELISAs.

1-Plex (IP-10) Magnetic BBA (Flowchart # 6)

Single-plex assay of IP-10 was determined in the NS tears of six subjects (normal: 1, 2, 5; dry: 20, 21, and 23) and the STIM tears of two subjects (1 and 2). Parallel samples were collected and stored in TAB and BBS (see Table 6) at -80°C. For each buffer, duplicate wells of NS and STIM were assayed for two subjects (1 and 2) and single wells of NS for the other four subjects (5, 20, 21, and 23). Linearity of dilution was also measured. Linearity of dilution determines the precision of an assay at various levels of dilution. For example, if a sample is diluted in half, then the resulting cytokine concentration should be half of the original sample concentration. The Bio-Rad 1-Plex magnetic BBA kit was used on a filter plate with single automatic wash steps.

3-Plex Magnetic BBA (Flowchart #7)

A 3-Plex magnetic BBA of IP-10, IL-8, and IFN-γ was completed on the NS and STIM tears of two normal subjects (1 and 2). Parallel samples were collected and stored

in TAB, DAB, and BSD (see Table 6) at -80°C. For each buffer, duplicate wells of NS and STIM were assayed for the two subjects. The Bio-Rad 3-Plex magnetic assay kit was used on a filter plate with the single automatic wash steps.

11-Plex Magnetic BBA (Flowchart #8)

NS and STIM tear samples stored in either TAB or DAB (see Table 6) were run on a Bio-Rad 11-Plex magnetic BBA kit using a filter plate and single automatic wash steps. NS and STIM tears were collected in each buffer for four subjects (normal: 1 and 2; dry: 21 and 24) and stored at -80°C. For each buffer, duplicate wells of NS and STIM were assayed for each subject. Cytokine concentration was compared for each buffer.

TAB with Anti-Protease (Flowchart #9)

In order to prevent post-translational breakdown of protein by proteases in tear samples, SigmaFast Protease Inhibitor Cocktail Tablet was added to TAB. A Bio-Rad 27-Plex polystyrene kit was used to assay STIM tear samples separately stored in TAB and TAB with anti-protease (TAB-AP) (see Table 6) at -80°C from Subject 21 (dry eye). Duplicate STIM samples were assayed in each buffer for the subject. This assay used a filter plate and single automatic wash steps.

Comparison of Tear Sample Types in TAB (Flowchart #10)

NS, WO, and STIM tear samples were all stored in TAB to determine if TAB is a valid buffer for each tear sample type. The NS, WO, and STIM tear samples were col-

lected in succession. NS were collected in 10-minute increments, WO in 5-minute increments, and STIM were collected until sufficient volume was obtained. The goal was to assay one NS, two WO, and duplicate STIM tear samples for 14 subjects (normal: 1, 2, 3, 4, 5, 6, 7, 8, 14; dry: 15, 17, 18, 21, and 23). All 14 subjects provided one NS and at least one WO tear sample. Only 10 were able to provide a second WO tear sample and 13 provided STIM tear samples. The samples were stored at -80°C and run with a Bio-Rad 27-Plex magnetic BBA kit. A filter plate and single automatic wash steps were used.

Linearity of Dilution and Spike-recovery (Flowchart #11)

Sensitivity and possible interference were determined by comparing linearity of dilution and spike-recovery on a Bio-Rad 27-Plex polystyrene and 27-Plex magnetic BBA. Spike-recovery involves adding standard solution to tear samples. The final concentration of this total sample should be equivalent to individual tear sample concentration plus standard sample concentration. Ideally, the spiked sample would give 100% recovery, which means there is likely no interference. If there was greater than 100% recovery, then some form of interference was amplifying the signal. The opposite was the case if the recovery was less than 100% with an interfering source blocking binding. Tear samples were diluted 1 in 8 and spiked with five times the concentrations of the appropriate standards. When mixed with tears in the ratio of one part 5x standard and four parts 1 in 8 tears, the result was 1 in 10 tears diluted with a given concentration of either Standard 7, 5, or 3. High spike contained Standard 7 in tears, medium spike contained Standard 5 in tears, and low spike contained Standard 3 in tears. STIM tear samples from

Subject 1 (normal) were pooled and stored in TAB-AP. Another comparison of linearity and spike-recovery was completed for the NS and STIM tear samples of Subjects 15 (dry) and 23 (dry) with polystyrene and magnetic BBAs. Filter plates and the automatic wash system with single wash steps were used for both assay types.

Final Assay Comparisons (Flowchart #12)

A Bio-Rad 27-Plex polystyrene and 27-Plex magnetic BBA were performed. Optimal techniques and conditions, which included a filter plate, automatic wash/aspiration system, and TAB-AP, were used for these assays. WO and STIM tear samples were obtained from 20 subjects (normal: 1, 2, 4, 5, 6, 7, 8, 9, 10, 11, and 12; dry: 15, 16, 17, 19, 20, 21, 22, 23, and 24). The STIM tear samples were assayed in duplicate. NS and WO tear samples were collected sequentially from six subjects (9, 11, 13, 19, 21, and 24). All samples of a specific tear type for each subject were pooled in order to give a true comparison of like samples.

Statistical Approach

Bio-Plex Manager software, which was connected directly to the Luminex instrument, was used to determine cytokine concentration, standard curves, limits of detection (LOD), limits of quantification (LOQ), and bead aggregation. Other statistical values were calculated using Microsoft Excel or SigmaPlot.

RESULTS

Performance of the Luminex System

In the assay used to determine how well the Luminex bead-based assay analysis system performed, several variables were assessed. This assay included a comparison of tear samples stored in the buffers MAB and MSB and determination of whether this assay's results were comparable to previously validated assays. The main variable evaluated was the overall system performance when compared to previous studies.

System Performance (Flowchart #1)

As part of the system performance evaluation, two standard and sample diluting buffers were compared using a Linco 13-Plex High Sensitivity polystyrene BBA kit. For each buffer, stimulated tear samples from seven subjects (normal: 1, 2, and 5; dry: 15, 17, 21, and 23) were run in quadruplicate. A 5-parameter logistic algorithm was used as the default curve fitting method for each standard curve. Tear (unknown) sample cytokine concentrations were determined by standard curve interpolation. Comparing the two buffers, assay performance was significantly better with MAB (Table 7). Table 8 shows that all cytokines except IL-4 were quantifiable (levels above the lower limit of quantification) in the majority of MAB-diluted tear samples. Conversely, this was the case for only five of the 13 cytokines when tears were diluted in MSB.

Table 7

Linco 13-Plex High Sensitivity Polystyrene BBA

Cytokine	MAB	SE	MSB	SE
IL-1β	1.96	0.54	-	-
IL-2	128.74	29.49	4.31	0.63
IL-4	2.67	-	-	-
IL-5	0.19	0.04	0.51	0.03
IL-6	12.38	3.73	10.49	4.76
IL-7	274.46	44.85	669.91	95.45
IL-8	108.10	7.87	47.42	5.33
IL-10	28.59	11.32	1.42	0.62
IL-12p70	5.39	1.68	-	-
IL-13	4.94	1.20	3.06	-
GM-CSF	4.41	1.61	-	-
IFN-γ	15.20	4.70	2.58	1.44
TNF-α	11.04	3.19	1.23	0.19

Tear cytokine concentrations in pg/mL. BBA = bead-based assay. MAB = Millipore assay buffer. MSB = Millipore serum matrix. SE = standard error.

Table 8

Number of Tear Samples in which Cytokines were Quantified or Detected: MAB and MSB

	MAB			MSB		
Cytokine	Below LOD	Below LLOQ	In Working Range	Below LOD	Below LLOQ	In Working Range
IL-1β	0	0	7	7	0	0
IL-2	0	0	7	0	0	7
IL-4	5	2	0	7	0	0
IL-5	0	0	7	0	0	7
IL-6	0	0	7	1	0	6
IL-7	0	0	7	0	0	7
IL-8	0	0	7	0	0	7
IL-10	1	0	6	5	1	1
IL-12p70	1	0	6	7	0	0
IL-13	1	2	5	6	0	1
IFN-γ	0	0	7	7	0	0
GM-CSF	2	1	4	7	0	0
TNF-α	0	0	7	6	0	1

MAB = Millipore assay buffer. MSB = Millipore serum matrix. LOD = limit of detection. LLOQ = lower limit of quantification.

Assay performance differed considerably for several cytokines, including IL-10. Figures 3a and 3b compare assay performance for this cytokine in terms of where the tear sample IL-10 concentrations were interpolated from the standard curve. More tear samples (green triangles) were above the assay LLOQ with MAB (Figure 4a) than with MSB (Figure 4b) despite the fact that both standard curves produced a similar LLOQ.

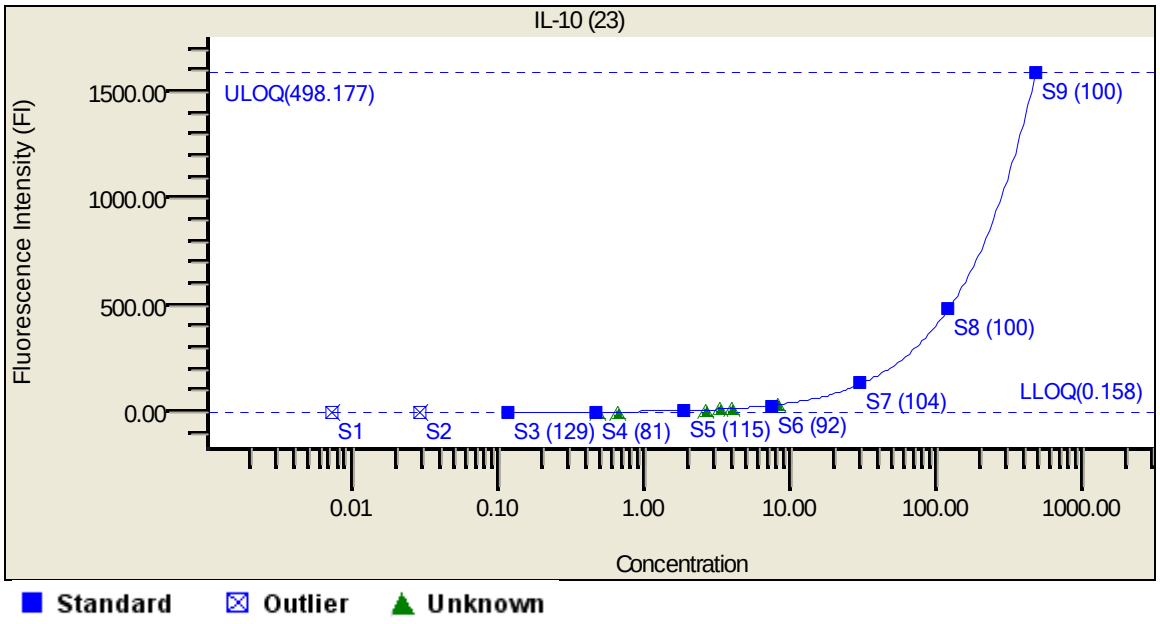

Figure 4a. Millipore assay buffer (MAB): IL-10 Standard Curve. Shows tear sample interpolation points (unknown, green triangle) on the standard curve.

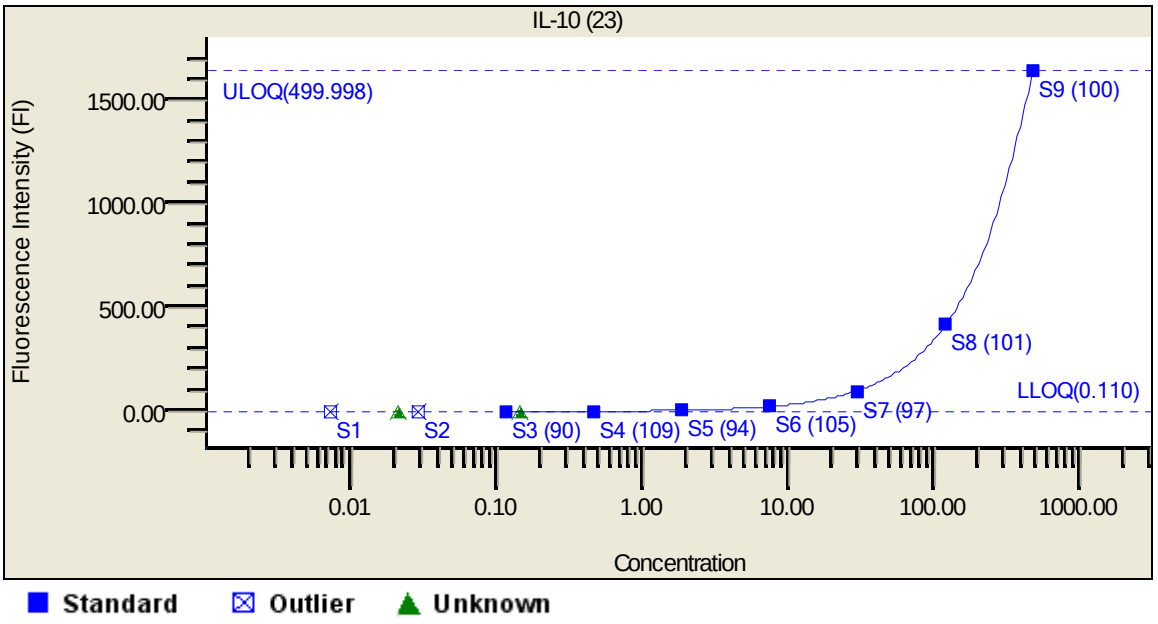

Figure 4b. Millipore serum matrix (MSB): IL-10 Standard Curve. Shows tear sample interpolation points (unknown, green triangle) on the standard curve.

Overall, assay results with MAB showed the Luminex system to be operating within required specifications. Twelve of 13 cytokines were detected in the majority of subjects (Table 8) in ranges consistent with previous studies conducted in this lab [46]. This indicates that all current assay steps were executed correctly and that the Luminex system performance was satisfactory. It was concluded that future assays could be successfully completed on the Luminex system.

Selection of Washing System

After the Luminex system was shown to be operating properly, the manual and automatic wash systems were compared to determine if the two provided similar results. Comparison of the wash systems was the only comparison in this section of the study.

Wash Systems (Flowchart #2)

For both wash systems, only four standards (chosen from the expected mid-region of assay working range for most cytokines) were run instead of the usual nine because this was intended as a simple check of consistency between washing methods rather than a full analysis of tear cytokine levels. Minimally stimulated tears from Subject 21 (dry eye) were run in triplicate on separate filter plates using a Bio-Rad 27-Plex polystyrene BBA kit. Both wash systems showed some cytokines that were either below (<OOR) of above (>OOR) the assay detection limit. The manual wash/vacuum plate had four cytokines outside assay detection range (three <OOR and one >OOR) and the automatic had five cytokines (four <OOR and one >OOR) not detected by the assay (Table 9). While

coefficients of variation (CV) showed differences between wash methods, CVs were not systematically lower for either system.

While the decision to run only four standards appeared to be reasonable, given the nature of the comparison being made, it did highlight the advantages of running a full standard curve instead of a partial curve. With only four standards, the standard curve could not be fit using a 4- or 5-parameter logistic function, so a simple log-log function was required. Fewer cytokines fell within the assay working range (between upper and lower limits of quantification) because the LLOQ and ULOQ range was considerably restricted. IL-6 was one cytokine for which the tear sample data fell nicely within the limits of quantification for both the manual and automatic wash systems (Figures 5a and 5b). On the other hand, IL-17 was outside of the limits for both wash systems (Figure 5c and 5d). IL-17 had a narrower range between the LLOQ and ULOQ for automatic than for manual. However, in both cases the tear interpolation points for IL-17 were below the limit of quantification of the standard curve. Overall, the automatic and manual systems performed similarly for the 27 cytokines.

Table 9

27-Plex Polystyrene BBA using Teknova Assay Buffer (TAB)

	Manual Wash/Vacuum		Automatic Wash/Aspiration	
Cytokine	Mean	CV%	Mean	CV%
IL-1β	>OOR	-	>OOR	-
IL-1ra	2,091.0 ↑	1.4	2,314.8 ↑	2.8
IL-2	49.4	24.6	24.6	5.8
IL-4	17.6	29.0	11.7	12.2
IL-5	7.6	32.5	10.6 ↓	4.6
IL-6	32.4	16.7	23.7	8.7
IL-7	141.1	6.8	158.2	12.7
IL-8	115.5	2.9	125.6	7.4
IL-9	11.7	10.9	6.1	32.7
IL-10	115.6	1.7	119.4	11.9
IL-12p70	48.3	7.2	49.1	12.5
IL-13	15.4	24.6	16.2	12.1
IL-15	<OOR	-	<OOR	-
IL-17	4.4 ↓	28.7	4.0	95.1
Eotaxin	27.9	19.9	<OOR	-
FGF basic	<OOR	-	<OOR	-
G-CSF	71.2	1.1	67.5	6.5
GM-CSF	56.6	30.1	22.0	16.0
IFN-γ	378.3 ↑	14.1	246.1	8.4
IP-10	18,618.5 ↑	4.5	24,219.9 ↑	4.2
MCP-1	24.6	23.5	29.8	27.8
MIP-1α	34.6	22.5	29.9	9.2
MIP-1β	<OOR	-	<OOR	-
PDGF-bb	21.7	12.5	16.7	17.7
RANTES	163.8	15.6	109.6	6.0
TNF-α	207.8	30.4	120.6	10.0
VEGF	421.0 ↑	4.6	507.9 ↑	21.8

Tear cytokine concentrations in pg/mL. BBA = bead-based assay. CV = coefficient of variation. <OOR = out of range (below). >OOR = out of range (above). ↑ = Above Upper Limits of Quantification (ULOQ). ↓ = Below Lower Limits of Quantification (LLOQ).

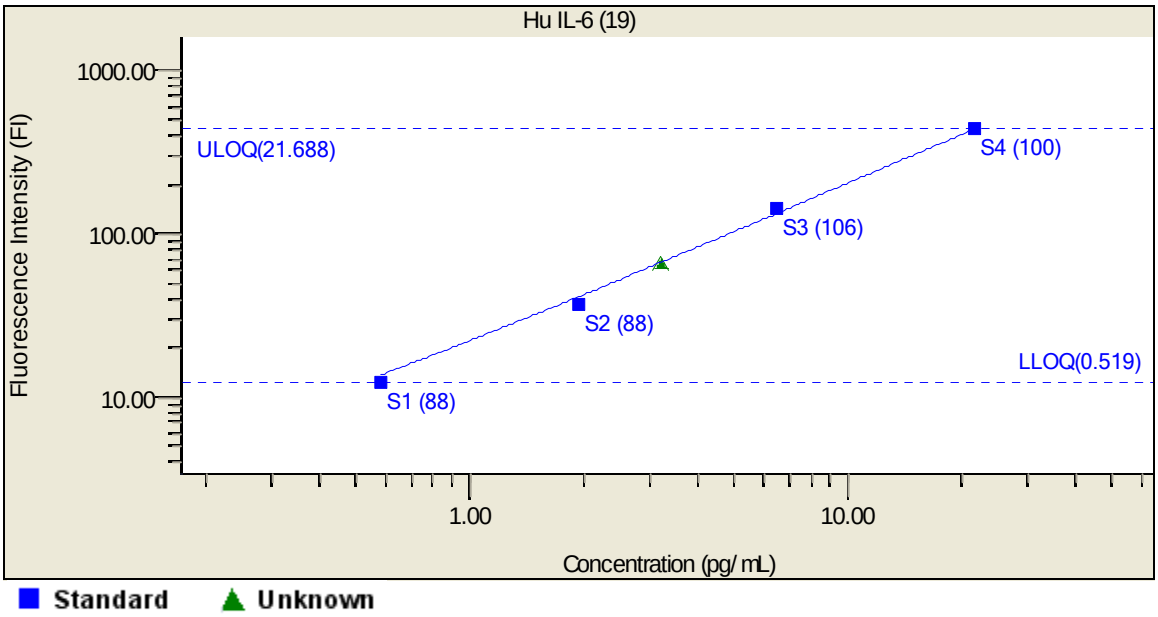

Figure 5a. Manual Wash/Vacuum System: IL-6 Standard Curve. Shows tear sample interpolation point (green triangle) on the standard curve. Standard curve correlation coefficient: 0.9992.

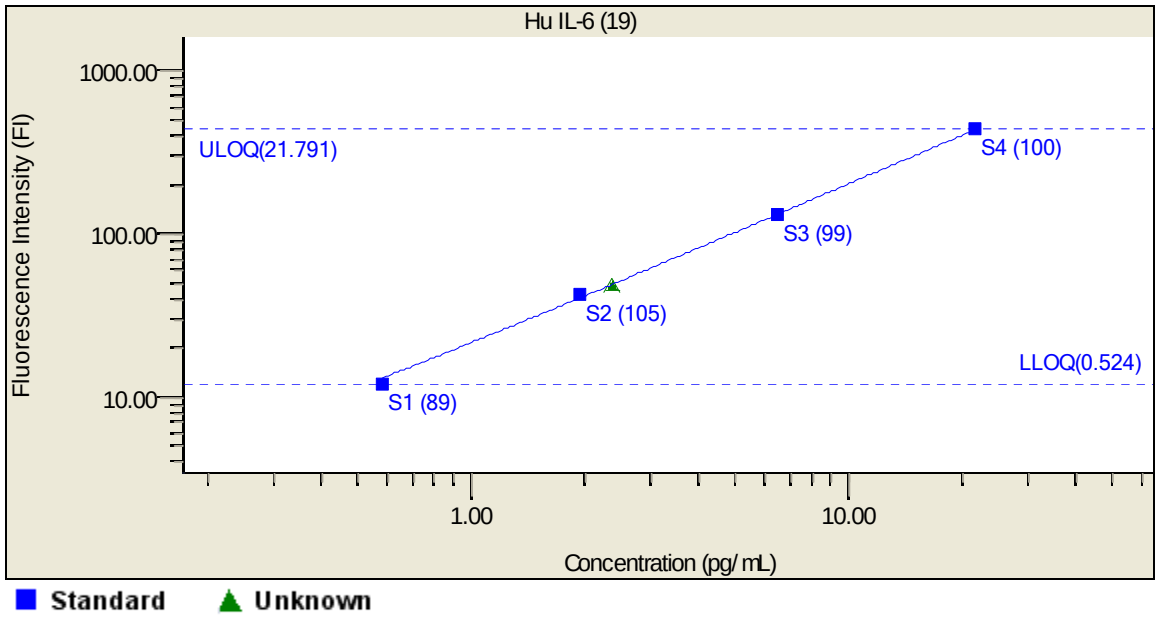

Figure 5b. Automatic Wash/Aspiration System: IL-6 Standard Curve. Shows tear sample interpolation point (green triangle) on the standard curve. Standard curve correlation coefficient: 0.9999.

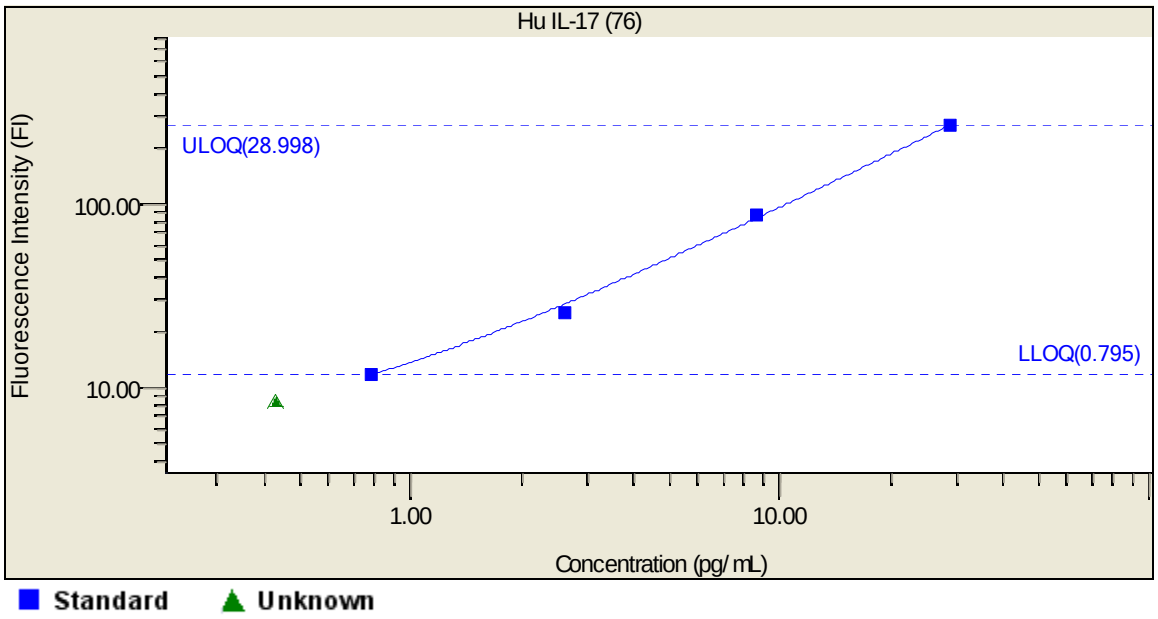

Figure 5c. Manual Wash/Vacuum System: IL-17 Standard Curve. Shows tear sample interpolation point (green triangle) on the standard curve. Standard curve correlation coefficient: 0.9995.

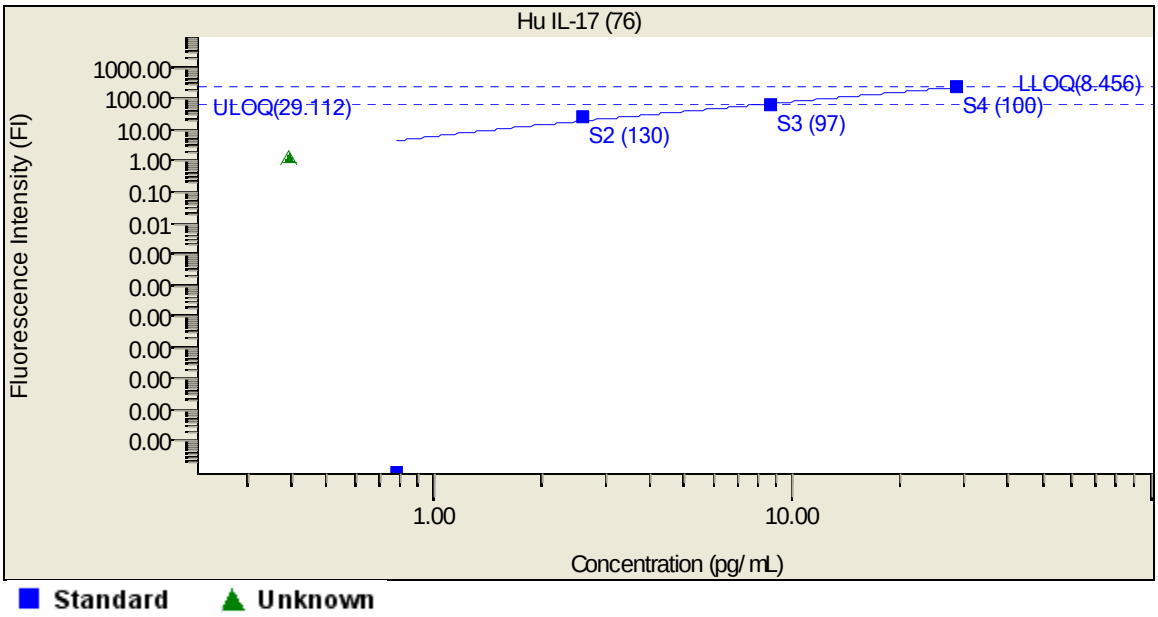

Figure 5d. Automatic Wash/Aspiration System: IL-17 Standard Curve. Shows tear sample interpolation point (green triangle) on the standard curve. Standard curve correlation coefficient: 0.9980.

The automatic wash/aspiration system was chosen as the system to use for future assays because the manual system did not show systematically superior performance. Manual vacuum aspiration of filter plates can produce well "dropouts" due to excessive vacuum pressure on individual wells and bead loss. This issue is eliminated with the automatic washer because it provides a constant vacuum across the entire plate.

Selection of Buffer: Polystyrene BBA (Flowchart #3 & 4)

To evaluate the effect of buffers on tears, various buffers were compared using the validated polystyrene BBA. These buffers contained BSA (TAB), were of proprietary composition (MAB and BAB), or contained high levels of animal serum (BBS). Animal serum was an important buffer component because it is known for reducing interference in assays [44, 45].

Comparison of TAB, MAB, and BAB (#3)

In this study, tear samples diluted in TAB, MAB, and BAB were assayed using a Bio-Rad 27-Plex polystyrene BBA kit. Standard curves were run in the three different buffers and quadruplicate aliquots of a STIM tear sample (Subject 15- dry eye) were run in each buffer. For 23 of 27 cytokines, the ascending order of concentration is from TAB to MAB to BAB (Table 10). MAB, which has been used in this lab in the past for tear sample storage, is more similar to TAB but still greater than TAB in concentration, in most cases. BAB had concentrations greater then MAB for many cytokines.

An interesting effect was seen with the BAB compared to the other two buffers (Table 11). In many cases, the LLOQ was considerably higher than for the other two buffers and the interpolated value for tear cytokine level was higher. BAB is the buffer supplied with the Bio-Rad kit. It is the only one of the three tested buffers that does not contain BSA. BSA is used as a blocking agent to reduce non-specific binding of proteins to solid surfaces. It is therefore apparent that BAB is producing a strong matrix effect, allowing greater non-specific binding of cytokine to the assay beads. This would reduce assay sensitivity and potentially produce less reliable values for tear cytokine levels – in this case, levels typically higher than with the other two buffers.

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Table 10a

27-Plex Polystyrene BBA Comparing TAB, MAB, and BAB (Stimulated Tears)

Cytokine	TAB	SE	MAB	SE	BAB	SE
IL-1β	15.83	6.36	19.41	3.03	35.4	3.08
IL-1ra	440.86	218.89	999.31	101.45	1,638.56	202.59
IL-2	68.12	30.77	135.38	23.76	197.84	13.84
IL-4	24.84	9.18	45.91	7.58	75.22	4.68
IL-5	26.09	8.7	46.05	8.38	101.11	5.36
IL-6	34.79	14.39	91.63	15.06	167.05	8.35
IL-7	59.24	27.96	106.46	6.94	781.9	59.75
IL-8	44.1	21.19	99.35	14.42	136.95	9.48
IL-9	71.68	39.97	160.84	15.77	248.55	29.03
IL-10	57.83	32.16	186.08	7.88	214.3	9.44
IL-12p70	43.56	20.36	82.56	9.59	172.19	9.33
IL-13	31.09	13.67	57.37	8.87	135.7	10.49
IL-15	5.35	2.58	7.81	0.63	50.31	5.56
IL-17	41.87	21.79	87.85	9.54	427.18	34.97

Tear cytokine concentrations in pg/mL. BBA = bead-based assay. SE = standard error. TAB = Teknova assay buffer . MAB = Millipore assay buffer. BAB = Bio-Plex assay buffer.

Table 10b

27-Plex Polystyrene BBA Comparing TAB, MAB, and BAB (Stimulated Tears)

Cytokine	TAB	SE	MAB	SE	BAB	SE
Eotaxin	105.05	54.57	395.34	45.97	360.91	23.34
FGF basic	92.21	15.22	177.97	28.90	116.69	10.94
G-CSF	44.23	17.50	87.82	11.04	203.92	11.85
GM-CSF	216.05	102.96	379.27	76.43	621.83	65.46
IFN-γ	883.90	387.09	1,299.82	257.64	11,191.45	840.88
IP-10	4,893.52	2,727.23	7,791.01	542.90	65,685.51	2,859.87
MCP-1	14.56	7.95	32.87	2.39	64.05	6.58
MIP-1α	89.72	40.21	258.79	22.93	267.62	12.72
MIP-1β	0	-	-	-	37.93	-
PDGF-bb	12.87	6.14	18.63	0.82	104.72	7.21
RANTES	125.11	47.05	346.86	46.62	315.22	14.72
TNF-α	573.37	303.97	1,007.68	194.54	838.53	87.88
VEGF	180.01	13.11	268.87	25.67	942.83	75.40

Tear cytokine concentrations in pg/mL. BBA = bead-based assay. SE = standard error. TAB = Teknova assay buffer. MAB = Millipore assay buffer. BAB = Bio-Plex assay buffer.

Table 11

Quantification Limits for TAB, MAB, and BAB for 27-Plex Polystyrene BBA

	TAB		MAB		BAB	
Cytokine	LLOQ	ULOQ	LLOQ	ULOQ	LLOQ	ULOQ
IL-1β	0.79	88.05	0.81	339.10	2.74	79.52
IL-1ra	1.59	23,864	6.31	26,186	18.06	26,121
IL-2	0.32	4,749.60	0.27	1,081	3.61	4,132
IL-4	0.14	207.25	0.13	473.79	0.46	1,656
IL-5	0.79	350.14	0.83	311.90	2.63	373.85
IL-6	0.56	877.80	0.56	11,509	1.74	864.66
IL-7	0.89	381.63	0.95	366.05	3.07	4,104
IL-8	0.65	985.73	0.61	11,772	7.29	11,550
IL-9	0.47	6,574	16.40	7,823	5.26	7,739
IL-10	0.50	841.38	0.54	9,092	1.73	739.55
IL-12p70	0.65	7,504	0.66	2,225	7.50	9,957
IL-13	0.85	389.01	0.90	369.10	9.94	13,006
IL-15	0.60	266.14	0.63	219.60	2.05	9,623
IL-17	0.75	11,199	26.59	14,936	2.39	12,616
Eotaxin	5.72	1,950	21.41	1,003	1.84	2,450
FGF basic	4.27	515.51	12.67	5,810	4.19	5,861
G-CSF	0.75	1,224	0.69	883.03	2.37	3,689
GM-CSF	0.21	3,736	8.34	3,656	2.82	3,647
IFN-γ	0.81	3,858	31.85	3,588	2.23	13,679
IP-10	1.81	2,532	1.81	705.77	6.04	6,028
MCP-1	0.59	1,049	0.46	640.92	2.03	290.91
MIP-1α	5.41	196.23	2.07	192.83	1.64	743.35
MIP-1β	5.19	192.63	17.93	181.65	2.08	669.76
PDGF-bb	0.61	905.54	0.66	923.04	7.44	9,647
RANTES	0.69	275.35	2.77	1,075.20	7.63	224.33
TNF-α	1.55	26,040	54.69	26,288	18.00	7,350
VEGF	8.26	13,448	0.75	16,001	2.76	3,327

Tear cytokine concentrations in pg/mL. BBA = bead-based assay. LLOQ = lower limit of quantification. ULOQ = upper limit of quantification. TAB = Teknova assay buffer. MAB = Millipore assay buffer. BAB = Bio-Plex assay buffer.

Comparing results for TAB and MAB provides the first indication that TAB is not equivalent to MAB. However, TAB did not demonstrate superior assay performance in all aspects, producing higher variability in tear cytokine levels than the other two buffers. This indicated that further buffer studies should be conducted prior to the transition to magnetic bead testing.

TAB versus BBS (#4)

In addition to buffer comparisons, linearity of dilution for tear sample volumes down to 1 μL was tested on a Bio-Rad 27-Plex polystyrene BBA kit. Stimulated tears from two normal subjects were diluted in TAB (Subjects 1 and 2 – duplicate wells/subject) and BBS (Subject 1 – duplicate wells). The BBS buffer generally produced higher LLOQs relative to TAB (Table 12), in some cases the difference exceeding two orders of magnitude. However, TAB occasionally produced a substantially higher LLOQ. Linearity of dilution data for subject 2 was run only with TAB, but showed good recovery (within 30% of 100%) down to a 2 μL tear volume for 10 cytokines (Table 13). Tear samples for Subject 1 were tested for linearity of dilution using both buffers. For each buffer, five cytokines showed good recovery down to 1.25 μL. Interestingly, these were mutually exclusive groups of five cytokines. Linearity was clearly superior with TAB. In fact, 13 of the 27 cytokines were not even detected at the 2.5 uL level with BBS. Conversely, all 27 cytokines were quantified down to the l.25 uL level with TAB.

Table 12

27-Plex Polystyrene BBA: Limits of Quantification (TAB and BBS)

	TAB		BBS	
Cytokine	LLOQ	ULOQ	LLOQ	ULOQ
IL-1β	0.83	330.43	0.78	949.30
IL-1ra	1.66	25,003.49	17.30	24,696.04
IL-2	0.27	1,288.68	1.19	4,986.46
IL-4	0.14	552.46	8.65	2,187.00
IL-5	0.83	333.01	0.77	182.21
IL-6	0.57	10,223.09	2.58	8,854.12
IL-7	0.95	5,617.47	10.71	14,821.74
IL-8	0.57	11,918.42	0.59	8,876.94
IL-9	1.57	7,528.77	2.18	7,265.92
IL-10	0.56	8,141.22	7.98	8,635.02
IL-12p70	0.66	10,410.27	0.63	10,155.00
IL-13	0.91	5,157.36	4.02	14,616.43
IL-15	0.64	9,830.97	0.64	8,316.27
IL-17	0.71	13,660.91	0.57	11,812.86
Eotaxin	5.02	9,518.07	125.67	8,837.96
FGF basic	42.71	1,433.21	0.27	4,907.78
G-CSF	0.75	2,557.63	0.74	11,494.43
GM-CSF	0.76	3,771.21	17.75	3,718.46
IFN-γ	0.93	3,276.62	202.10	13,679.91
IP-10	1.86	2,847.52	29.31	28,493.83
MCP-1	0.69	2,159.31	0.62	2,896.47
MIP-1α	4.61	837.39	1.75	114.47
MIP-1β	0.39	1,597.65	1.64	6,097.35
PDGF-bb	0.71	2,639.37	560.84	10,346.79
RANTES	0.68	11,743.57	2.33	9,914.10
TNF-α	15.87	25,218.99	1.86	23,196.32
VEGF	0.97	13,655.91	13.11	13,958.68

Tear cytokine concentrations in pg/mL. BBA = bead-based assay. TAB = Teknova assay buffer. BBS = Brookwood Biomedical serum buffer. LLOQ = lower limit of quantification. ULOQ = upper limit of quantification.

Table 13a

27-Plex Polystyrene BBA: Linearity of Dilution (TAB and BBS)

		IL-1β	IL-1ra	IL-2	IL-4	IL-5	IL-6	IL-7	IL-8	IL-9	IL-10
Subject 2	5 µL level	0.75	632.03	10.38	3.93	0.90	33.95	15.82	139.85	34.88	95.98
TAB	4 µL Rec.	106.7%*	92.7%	98.1%	88.3%	92.2%	90.3%	86.9%	96.4%	103.7%	106.0%
	3 µL Rec.	134.7%	100.3%	97.0%	93.4%	105.6%	93.6%	98.2%	100.4%	126.6%*	119.8%*
	2 µL Rec.	169.3%	119.7%*	108.6%*	74.8%*	74.4%	90.8%	103.7%*	107.8%*	171.6%	135.7%
	1 µL Rec.	364.0%	144.7%	132.5%	47.3%	177.8%	97.9%*	133.8%	133.0%	316.1%	216.3%
Subject 1	5 µL level	1.70	6,734.54	56.31	21.84	8.09	26.23	86.66	160.47	65.56	103.71
TAB	2.5 µL Rec.	167.1%	111.9%	133.0%	141.3%	127.9%	120.1%	107.3%	115.2%	155.0%	156.2%
	1.25 µL Rec.	277.6%	113.9%*	164.9%	174.1%	107.4%*	121.7%*	103.1%*	120.8%*	245.0%	186.8%
Subject 1	5 µL level	1.21	30.35	-	46.16	2.56	6.7	11,306.6	256.24	233.81	4,611.95
BBS	2.5 µL Rec.	173.6%	-	-	-	-	-	28.5%	130.3%	105.2%	109.5%
	1.25 µL Rec.	381.8%	-	-	-	-	-	17.0%	147.1%	108.5%*	113.2%*

Non-stimulated tears. Tear cytokine concentrations (5 µL sample) in pg/mL. BBA = bead-based assay. Rec. = recovery. *Indicates lowest volume at which linearity of dilution recovery was within 30% of the 5 µL value. TAB = Teknova assay buffer. BBS = Brookwood Biomedical serum buffer.

Table 13b

27-Plex Polystyrene BBA: Linearity of Dilution (TAB and BBS)

		IL-12p70	IL-13	IL-15	IL-17	Eotaxin	FGF basic	G-CSF	GM-CSF	IFN-γ	IP-10
Subject 2	5 µL level	25.18	9.41	13.11	17.63	106.50	21.72	1.82	44.46	32.20	6,035.05
TAB	4 µL Rec.	93.0%	106.1%	91.6%	111.2%*	110.4%	92.1%	24.7%	97.6%	112.8%	95.4%
	3 µL Rec.	109.3%*	126.9%*	101.9%	141.2%	126.0%*	95.1%*	5.5%	96.4%	117.0%	112.3%*
	2 µL Rec.	133.8%	182.5%	109.5%*	222.5%	159.8%	153.9%	-	99.3%	108.3%*	134.6%
	1 µL Rec.	162.3%	212.8%	168.1%	478.0%	264.1%	284.6%	-	129.31%*	159.3%	181.7%
Subject 1	5 µL level	32.30	11.06	11.19	34.53	135.76	48.74	14.65	99.89	341.27	24,217.22
TAB	2.5 µL Rec.	134.6%	138.4%	153.4%	194.8%	154.0%	231.4%	170.3%	121.1%	125.3%*	142.7%
	1.25 µL Rec.	177.0%	160.3%	248.3%	341.9%	250.1%	476.1%	212.6%	128.5%*	140.1%	121.0%
Subject 1	5 µL level	79.51	101.47	-	10.78	-	-	94.42	-	-	345,429.70
BBS	2.5 µL Rec.	114.2%	131.7%	-	-	-	-	54.4%	-	-	106.7%
	1.25 µL Rec.	114.8%*	143.1%	-	-	-	-	98.6%*	-	-	102.3%*

Non-stimulated tears. Tear cytokine concentrations (5 µL sample) in pg/mL. BBA = bead-based assay. Rec. = recovery.*Indicates lowest volume at which linearity of dilution recovery was within 30% of the 5 µL value. TAB = Teknova assay buffer. BBS = Brookwood Biomedical serum buffer.

Table 13c

27-Plex Polystyrene BBA: Linearity of Dilution (TAB and BBS)

		MCP-1	MIP-1α	MIP-1β	PDGF-bb	RANTES	TNF-α	VEGF
Subject 2	5 µL level	701.70	52.76	100.53	7.41	40.86	127.49	254.05
TAB	4 µL Rec.	91.5%	115.8%*	114.2%*	90.4%	95.3%	93.4%	93.1%
	3 µL Rec.	103.6%	145.2%	146.9%	115.8%*	107.9%	104.8%*	107.8%
	2 µL Rec.	111.8%	208.2%	211.2%	150.6%	101.1%*	131.5%	127.0%*
	1 µL Rec.	129.7%*	390.6%	430.9%	241.4%	97.3%	221.6%	178.0%
Subject 1	5 µL level	60.26	110.14	182.45	16.53	156.53	346.84	376.56
TAB	2.5 µL Rec.	154.5%	173.8%	191.9%	153.8%	148.8%	143.4%	139.0%
	1.25 µL Rec.	281.6%	290.2%	343.2%	226.0%	186.6%	194.2%	169.8%
Subject 1	5 µL level	1,135.45	11.17	-	38,741.93	173.71	-	3,592.53
BBS	2.5 µL Rec.	118.4%	105.5%*	-	138.2%	119.9%*	-	161.3%
	1.25 µL Rec.	102.1%*	-	-	178.3%	160.1%	-	185.8%

Non-stimulated tears. Tear cytokine concentrations (5 µL sample) in pg/mL. BBA = bead-based assay. Rec. = recovery. *Indicates lowest volume at which linearity of dilution recovery was within 30% of the 5 µL value. TAB = Teknova assay buffer. BBS = Brookwood Biomedical serum buffer.

Overall, the data suggested that TAB would be a more reliable choice for the polystyrene BBA. It was therefore chosen as a key buffer to pursue in subsequent magnetic bead assay studies.

Selection of Plate Type

The initial magnetic BBA investigation was a comparison of filter and plastic plates. A single-plex assay was chosen instead of a multiplexed assay in order to avoid any cross-reactions between multiple cytokines. IL-8 was selected because it has been reported to show interference in tear assays [35, 37].

Magnetic BBA Test: Filter Plate and Plastic Plate (Flowchart #5)

Previous 27-Plex magnetic BBAs run in this laboratory had demonstrated different results for plastic versus filter plates. In the initial magnetic BBA in the current study, a Bio-Rad single-plex IL-8 magnetic BBA was used to avoid any potential antibody-antigen cross-reaction from multiplexing. Given previous success with TAB as assay buffer in polystyrene BBAs, TAB was also used in this study. Six subjects participated. Two normal subjects (1 and 2) collected NS and stimulated tear samples that were run in duplicate and used for a linearity of dilution comparison between plates. The other four subjects (normal: 5; dry: 20, 21, and 22) collected single NS samples that were divided into matching aliquots for each plate. The assay showed some surprising results. Bead aggregation with the plastic plate in particular (Table 14) and very low bead counts for both plates were a significant issue. There was much greater bead aggregation with the plastic

plate when compared to the filter plate. Neither issue had occurred with all previous, polystyrene BBAs.

Table 14

IL-8 Magnetic Bead Aggregation on Filter and Plastic Plates (Tear Sample Wells)

	Non-stimulated	Stimulated
Filter	14.38	6.75
Plastic	70.00	65.75

Percentage values indicate percentage aggregated beads.

In addition, the filter plate produced a substantially lower LLOQ of 0.444 pg/mL (Figure 6a), compared with 16.70 pg/mL for the plastic plate (Figure 6b). IL-8 levels were consistently higher with the plastic plate (Table 15). Despite this, linearity of dilution was superior with the filter plate. For both subjects participating in the linearity of dilution, NS and stimulated tears produced satisfactory recovery down to 2.5 μL on the filter plate. None of the samples showed satisfactory recovery below 5 μL on the plastic plate.

The outcome of this initial magnetic BBA was to select filter plates for subsequent assays, to expand buffers beyond TAB, given the aggregation effect, and to investigate potential reasons for such low bead counts.

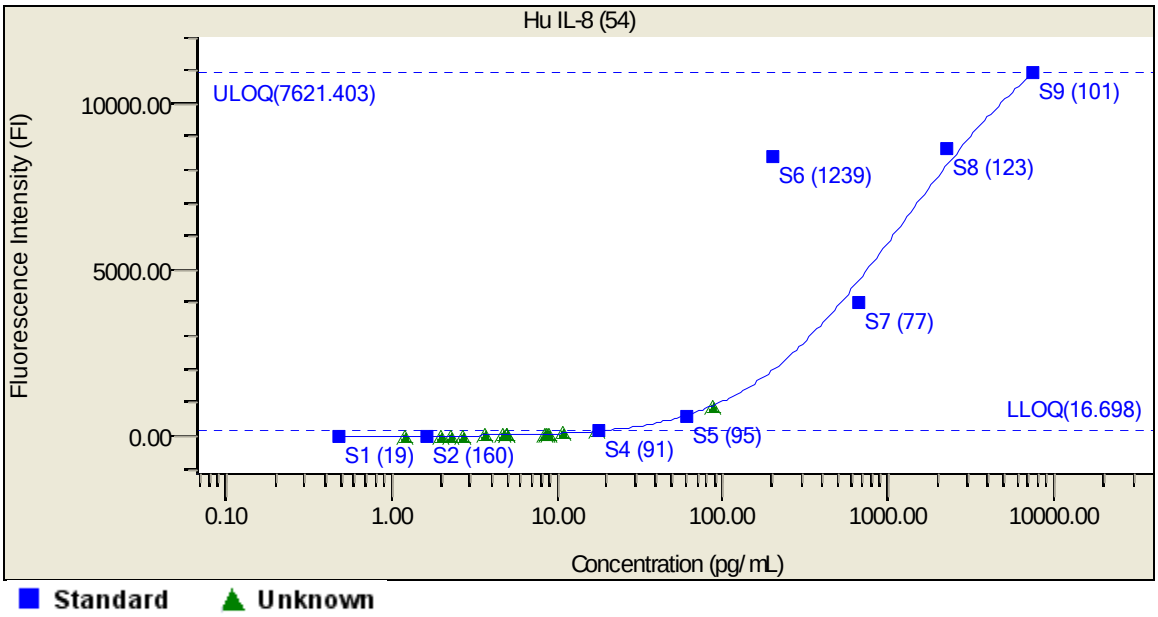

Figure 6a. Plastic Plate: Magnetic Bead IL-8 Single-Plex Standard Curve. Shows tear sample interpolation point (green triangle) on the standard curve.

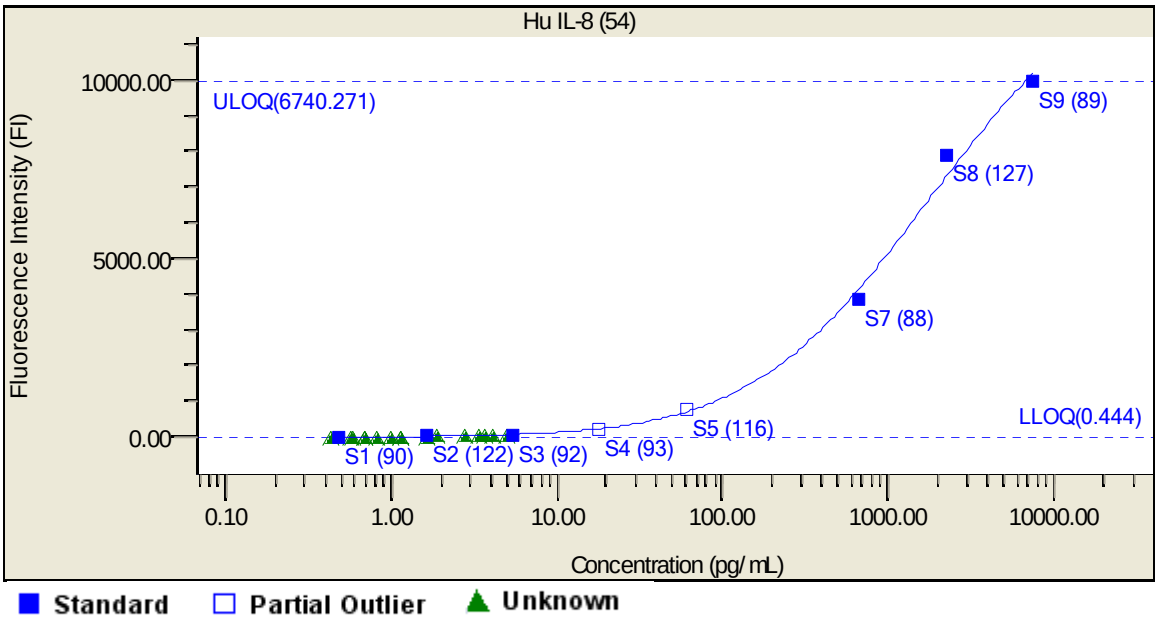

Figure 6b. Filter Plate: Magnetic Bead IL-8 Single-Plex Standard Curve. Shows tear sample interpolation point (green triangle) on the standard curve.

Table 15

Filter vs. Plastic Plate: IL-8 Magnetic BBA (Tear Levels and Linearity of Dilution)

	Filter Plate		Plastic Plate	
	Non-stimulated	Stimulated	Non-stimulated	Stimulated
5 µL level	11.46	16.44	27.8	107.42
2.5 µL Rec.	123.21%*	100.94%*	360.76%	67.33%
1.25 µL Rec.	246.42%	149.83%	173.83%	-
5 µL level	50.59	11.64	87.45	-
2.5 µL Rec.	111.94%*	100.30%*	194.55%	-
1.25 µL Rec.	149.83%	158.59%	217.14%	-
5 µL level	37.61		-	
5 µL level	33.95		177.43	
5 µL level	6.01		20.37	
5 µL level	42.02		91.33	

Tear cytokine concentrations (5 µL sample) in pg/mL. BBA = bead-based assay. Rec. = recovery. *Indicates lowest volume at which linearity of dilution recovery was within 30% of the 5 µL value.

Selection of Buffer: Magnetic BBA (Flowchart #6, 7, 8, 9, & 10)

After testing various buffers using the polystyrene BBA, some of the buffers already used in this study and new buffers were compared using the magnetic BBA. The number of cytokine types (assay "Plex") included in these magnetic buffer comparisons was gradually increased in order see if there were different effects based on the number of cytokine types assayed. Buffer selection was based on the presence or absence of animal serum, detergent, and anti-proteases. After determination of the optimal buffer, different tear sample types (NS, WO, and STIM) stored in that buffer were compared using a magnetic BBA.

1-Plex (IP-10) Magnetic BBA: Comparing TAB and BBS (Flowchart #6)

For the Bio-Rad IP-10 magnetic BBA, samples were diluted in TAB or BBS in order to compare concentrations and linearity of dilution of tear samples with each buffer. The IP-10 plate plan was very similar to that of IL-8. Six subjects participated. Two subjects (normal: 1 and 2) collected NS and stimulated tear samples that were run in duplicate and used for a linearity of dilution comparison between plates. The other four subjects (normal: 5; dry: 20, 21, and 23) collected single NS samples that were divided into matching aliquots for each plate. TAB showed a very different tear dose-response curve compared to BBS (Figures 7a and 7b). As a result, interpolated tear IP-10 levels were substantially higher with BBS (Table 16) and much higher than found in the earlier comparisons of polystyrene BBAs and ELISAs [35, 46]. This indicates significant matrix effects due to interactions between the magnetic beads and BBS-diluted tear samples. Despite the differences in absolute tear IP-10 levels, linearity of dilution results was similar for the two assay buffers. For subject 1, NS tears showed acceptable recovery down to 2.5 μL for Subject 1 with BBS. The only acceptable recovery of either buffer was down to 2.5 μL for NS tears diluted in BBS (Subject 1).

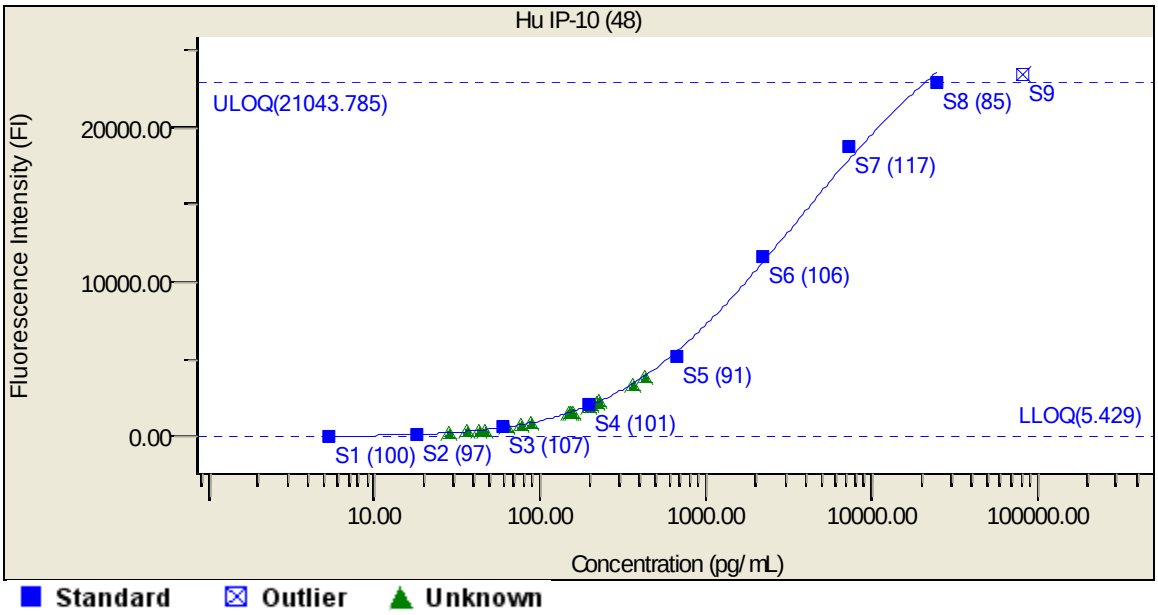

Figure 7a. Magnetic Bead IL-10 Single-Plex Standard Curve (TAB). Shows tear sample interpolation point (green triangle) on the standard curve.

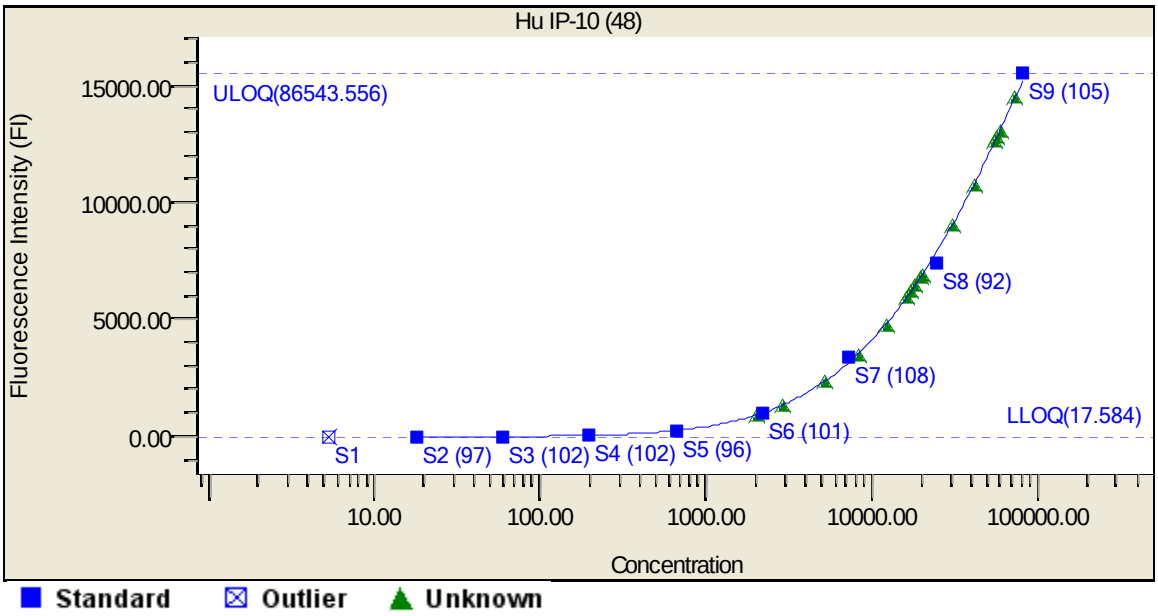

Figure 7b. Magnetic Bead IL-10 Single-Plex Standard Curve (BBS). Shows tear sample interpolation point (green triangle) on the standard curve.

Table 16

1-Plex Magnetic Bead (IP-10) BBA: Tear Levels and Linearity of Dilution Comparing TAB and BBS

		TAB		BBS	
		Non-stimulated	Stimulated	Non-stimulated	Stimulated
Subject 1	5 µL level	766	471	204,452	167,558
	2.5 µL Rec.	166.78%	181.23%	120.85%*	62.39%
	1.25 µL Rec.	192.78%	241.19%	57.66%	48.69%
Subject 2	5 µL level	2,215	1,517	184,789	309,286
	2.5 µL Rec.	185.84%	203.16%	456.74%	114.18%*
	1.25 µL Rec.	287.17%	233.66%	440.26%	108.11%*
Subject 5	5 µL level	2,307		561,196	
Subject 20	5 µL level	1,989		583,014	
Subject 21	5 µL level	4,292		749,174	
Subject 23	5 µL level	3,635		602,339	

Tear cytokine concentrations (5 µL sample) in pg/mL. BBA = bead-based assay. Rec. = recovery. *Indicates lowest volume at which linearity of dilution recovery was within 30% of the 5 µL value. TAB = Teknova assay buffer. BBS = Brookwood Biomedical serum buffer.

Comparing the IL-8 and IP-10 tear data obtained in these two magnetic BBAs (Tables 15 and 16) with ELISA and polystyrene BBA data compiled by other students in Dr. Fullard's lab (Table 17) [35] shows that TAB is providing results that are consistent with these earlier findings. In the earlier studies, IL-8 concentrations were approximately 2.5 times higher with ELISAs than polystyrene BBAs [35]. ELISA IP-10 concentrations using DAB [NS: 2,230.34; STIM: 1,265.52] are much closer to the current IP-10 data with TAB [NS: 2,276.18; STIM: 1,011.27] than BBS [NS: 407,344.37; STIM:

235,068.38]. Taken together, these findings cast doubt on the validity of data obtained with BBS as the sample diluent, presumably due to substantial matrix effects.

Table 17

IP-10 and IL-8 ELISA Concentrations [35]Compared to Magnetic 1P-10 and 3-Plex (IL-8, IFN-γ and IP-10) BBAs

	Buffer	IL-8	IP-10
Non-stimulated Tears	DAB	293.52	2,230.34
Stimulated Tears	DAB	79.36	1,265.52

Tear cytokine concentrations in pg/mL. BBA = bead-based assay. DAB = Diaclone standard diluent buffer.

3-Plex Magnetic BBA: IL-8, IFN-γ, and IP-10 (Flowchart #7)

Three sample and standard diluting buffers were used in this study of the Bio-Rad 3-Plex (IL-8, IFN-γ and IP-10) magnetic BBA: TAB, DAB, and BSD. NS and STIM tear samples were collected from normal subjects (1 and 2) and run in duplicate in each buffer for each subject. Tear levels and linearity of dilution results are shown in Table 18. For all three buffers, IL-8 levels were within the range found in previous polystyrene BBAs.

IFN-γ levels with TAB were in the 30 – 110 pg/mL range, which is lower than previously found in polystyrene BBAs (246 – 884 pg/mL). IFN-γ was almost ten times higher with DAB (299 – 1,058 pg/mL) than TAB and showed intermediate values with BSD (54 – 629 pg/mL). In percentage terms, IP-10 levels showed the least variability across the three buffers, but were lowest with TAB. Linearity of dilution results showed

acceptable recovery in 2.5 μL samples in three cases with TAB, six with DAB and four with BSD (Table 18).

Table 18

3-Plex (IL-8, IFN-γ, IP-10) BBA: Tear Levels and Linearity of Dilution Comparing TAB, DAB, and BSD

		IL-8		IFN-γ		IP-10	
		NS	STIM	NS	STIM	NS	STIM
	TAB						
Subject 1	5 μL level	30.86	19.78	31.18	79.25	1,502	924.7
	2.5 μL Rec.	111%*	-	-	-	164%	160%
Subject 2	5 μL level	53.11	47.28	108.69	176.25	1,967	1,652
	2.5 μL Rec.	28.1%	80.9%*	63.6%	98.8%*	257%	324%
	DAB						
Subject 1	5 μL level	37.60	44.82	298.57	660.12	4,752	3,124
	2.5 μL Rec.	115%*	89.3%*	177%	89.3%*	134%	126%*
Subject 2	5 μL level	46.06	63.92	1,041.0	1,057.6	7,089	6,749
	2.5 μL Rec.	117%*	143%	88.7%*	168%	183%	291%
	BSD						
Subject 1	5 μL level	40.38	28.33	53.60	225.13	5,087	3,503
	2.5 μL Rec.	191%	117%*	-	74%*	187%	210%
Subject 2	5 μL level	23.85	35.20	331.53	629.3	3,322	3,630
	2.5 μL Rec.	153%	124%*	50.52%	103%*	289%	353%

5 μL level concentrations in pg/mL. BBA = bead-based assay. Rec. = recovery.
*Indicates lowest volume at which linearity of dilution recovery was within 30% of the 5μL value. TAB = Teknova assay buffer. DAB = Diaclone standard diluent buffer. BSD = Bio-Plex human serum standard diluent.

As with the previous 1-Plex IP-10 magnetic BBA, the 3-Plex IP-10 results with TAB (Table 19) correlate with previous IP-10 levels found in comparisons of ELISA and polystyrene BBAs (Table 17). However, IP-10 levels measured in DAB and BSD exceeded the ELISA and polystyrene BBA range.

Table 19

Mean 5 μL NS and STIM Tear Levels in 3-Plex Magnetic BBA – Comparison of Three Buffers (TAB, DAB, and BSD)

	Buffer	IL-8	IFN-γ	IP-10
Non-stimulated	TAB	41.99	69.94	1,735
	DAB	41.83	669.8	5,921
	BSD	32.12	192.6	4,205
Stimulated	TAB	33.53	127.8	1,288
	DAB	54.37	858.9	4,937
	BSD	31.77	427.2	3,567

Tear cytokine concentrations in pg/mL. BBA = bead-based assay. TAB = Teknova assay buffer. DAB = Diaclone standard diluent buffer. BSD = Bio-Plex human serum standard diluent.

Overall results with these three assay buffers were inconclusive because no single buffer stood out as giving both the most reliable results and results most consistent with previous polystyrene bead-based tear assays.

11-Plex Magnetic BBA (Flowchart #8)

Comparison of TAB and DAB. NS and STIM tear samples from four subjects (normal: 1, 2; dry: 21, 24) were run in duplicate to evaluate the Bio-Rad 11-Plex magnet-

ic BBA using TAB and DAB as assay buffers. Linearity of dilution expressed as recovery of 2.5 μL tear volumes relative to 5 μL volumes demonstrated that TAB was superior to DAB (Tables 20 and 21). In 33 of 88 cases, TAB provided acceptable recovery at the 2.5 μL level, compared to 12 of 88 cases with DAB.

Different cytokine levels in matched TAB-diluted and DAB-diluted aliquots of the same tear sample were seen in almost all cases (Table 21a), the DAB levels usually being significantly higher by paired t-test (Table 21b). Plotting levels of each cytokine in the two normal subjects versus the two dry eye subjects showed that the overall trends in rank ordering of cytokine level were similar, but not identical with the two buffers. Plots show this trend in NS tears (Figure 8a) and STIM tears (Figure 8b).

Table 20a

11-Plex Magnetic BBA: Non-stimulated and Stimulated Tear Levels and Linearity of Dilution (TAB)

	IL-1β	IL-2	IL-4	IL-6	IL-8	IL-10	IL-12p70	IL-17	IFN-γ	IP-10	TNF-α
Sub 1 NS5µL	3.84	-	99.14	21.36	93.86	7.40	25.54	13.04	302.75	3,100	81.48
2.5 µL Rec.	185%	-	173%	132%	142%	156%	185%	110%*	143%	133%	181%
Sub 2 NS5µL	10.68	36.48	305.69	57.13	168.53	19.92	61.45	102.93	799.23	4,973	312.59
2.5 µL Rec.	155%	178%	146%	129%*	123%*	136%	148%	98.6%*	140%	147%	124%*
Sub 21 NS5µL	10.99	12.85	218.55	75.49	464.24	13.66	48.77	50.72	555.23	8,346	206.65
2.5 µL Rec.	77%*	-	86%*	107%*	134%	109%*	99%*	40%	84%*	160%	76%*
Sub 24 NS5µL	10.56	21.71	273.83	44.95	198.30	14.75	48.47	64.26	665.66	4,325	245.10
2.5 µL Rec.	113%*	16%	103%*	112%*	135%	128%*	139%	98%*	112%*	179%	112%*

5 µL level concentrations in pg/mL. BBA = bead-based assay. TAB = Teknova assay buffer. Sub. = subject. NS = non-stimulated tears. Rec. = recovery.

Table 20b

11-Plex Magnetic BBA: Non-stimulated and Stimulated Tear Levels and Linearity of Dilution (TAB)

	IL-1β	IL-2	IL-4	IL-6	IL-8	IL-10	IL-12p70	IL-17	IFN-γ	IP-10	TNF-α
Sub 1 ST5µL	13.32	42.43	346.79	66.53	162.78	20.86	76.03	112.10	919.29	912.91	340.10
2.5 µL Rec.	135%	66%	120%*	118%*	119%*	141%	135%	122%*	141%	162%	140%
Sub 2 ST5µL	15.13	55.65	395.16	74.22	190.68	24.54	87.08	156.62	1,094	2,735	408.95
2.5 µL Rec.	196%	163%	199%	184%	168%	169%	165%	158%	179%	183%	173%
Sub 21 ST5µL	10.98	43.26	320.84	54.33	140.87	14.66	50.93	85.06	653.04	2,348	232.30
2.5 µL Rec.	137%	19%	119%*	120%*	142%	172%	166%	97%*	153%	171%	159%
Sub 24 ST5µL	9.09	24.83	246.27	44.63	150.11	15.66	48.19	59.54	601.23	3,785	243.56
2.5 µL Rec.	134%	-	112%*	124%*	108%*	103%*	122%*	68%	121%*	188%	99%*

5 µL level concentrations in pg/mL. BBA = bead-based assay. TAB = Teknova assay buffer. Sub. = subject. ST = stimulated tears. Rec. = recovery.

Table 20c

11-Plex Magnetic BBA: Non-stimulated and Stimulated Tear Levels and Linearity of Dilution (DAB)

	IL-1β	IL-2	IL-4	IL-6	IL-8	IL-10	IL-12p70	IL-17	IFN-γ	IP-10	TNF-α
Sub 1 NS5µL	12.70	107.22	510.20	115.25	185.40	6.00	109.08	286.47	3,482	7,758	130.21
2.5 µL Rec.	51.1%	44.9%	83.3%*	73.4%*	103%*	-	74.3%*	65.2%	79.9%*	144%	102%*
Sub 2 NS5µL	33.20	218.03	952.12	245.24	270.36	53.85	211.50	587.31	7,112	12,732	273.70
2.5 µL Rec.	139%	151%	142%	135%	153%	115%*	157%	148%	160%	140%	147%
Sub 21 NS5µL	16.87	122.80	556.52	169.09	357.36	20.14	141.91	367.19	4,479	15,408	158.05
2.5 µL Rec.	145%	148%	169%	163%	176%	86%*	131%	148%	161%	206%	156%
Sub 24 NS5µL	22.57	142.57	602.27	149.23	245.99	23.71	136.29	393.00	5,229	13,593	172.38
2.5 µL Rec.	104%*	134%	141%	139%	150%	66%	148%	123%*	115%*	154%	139%

5 µL level concentrations in pg/mL. BBA = bead-based assay. DAB = Diaclone standard diluent buffer. Sub. = subject. NS = non-stimulated tears. Rec. = recovery.

Table 20d

11-Plex Magnetic BBA: Non-stimulated and Stimulated Tear Levels and Linearity of Dilution (DAB)

	IL-1β	IL-2	IL-4	IL-6	IL-8	IL-10	IL-12p70	IL-17	IFN-γ	IP-10	TNF-α
Sub 1 ST5µL	39.83	238.64	925.73	244.82	285.43	60.96	237.99	653.67	8,049	4,202	329.43
2.5 µL Rec.	135%	160%	175%	161%	162%	130%*	176%	149%	159%	173%	148%
Sub 2 ST5µL	32.91	195.57	819.90	217.30	245.55	47.09	181.83	559.81	6,903	10,265	241.49
2.5 µL Rec.	261%	288%	256%	317%	247%	319%	301%	263%	261%	193%	292%
Sub 21 ST5µL	22.70	163.72	723.11	189.84	239.25	38.59	171.82	467.97	5,899	9,159	206.91
2.5 µL Rec.	207%	211%	195%	192%	172%	174%	195%	201%	205%	174%	206%
Sub 24 ST5µL	30.59	209.87	790.71	208.72	284.25	36.56	181.99	486.59	6,116	16,542	229.68
2.5 µL Rec.	157%	160%	196%	186%	165%	188%	190%	178%	193%	146%	218%

5 µL level concentrations in pg/mL. BBA = bead-based assay. DAB = Diaclone standard diluent buffer. Sub. = subject. ST = stimulated tears. Rec. = recovery.

Table 21a

11-Plex Magnetic BBA: Differences in Cytokine Levels with TAB and DAB

	Non-stimulated Tears		Stimulated Tears	
Cytokine	TAB	DAB	TAB	DAB
IL-1β	10.44	25.62	15.07	43.86
IL-2	26.28	184.48	47.82	293.13
IL-4	258.97	823.68	393.44	1,188.9
IL-6	57.74	213.36	70.99	328.63
IL-8	288.87	353.07	189.64	363.26
IL-10	16.67	29.35	22.65	67.11
IL-12p70	55.80	188.50	78.51	285.97
IL-17	66.49	504.26	112.01	777.99
IFN-γ	664.87	6,420.3	993.00	9,837.1
IP-10	7,021.9	17,151.9	3,698.4	14,282.7
TNF-α	237.91	231.71	363.35	377.37

Tear cytokine concentrations in pg/mL. BBA = bead-based assay. TAB = Teknova assay buffer. DAB = Diaclone standard diluent buffer.

Table 21b

11-Plex Magnetic BBA: Differences in Cytokine Levels with TAB and DAB

	NS TAB vs. DAB			STIM TAB vs. DAB		
	t (or Z)	df	P value	t (or Z)	df	P value
IL-1b	-6.03	13	0.001**	-7.02	13	0.001**
IL-2	Z = 2.52	-	0.008*	-5.53	9	0.001**
IL-4	-9.12	13	0.001**	-7.4	13	0.001**
IL-6	Z = 3.30	-	0.001**	-6.41	13	0.001**
IL-8	-2.06	13	0.06	-6.42	13	0.001**
IL-10	-2.7	13	0.019*	-5.3	13	0.001**
IL-12p70	-8.33	13	0.001**	-6.91	13	0.001**
IL-17	-9.11	11	0.001**	-7.85	13	0.001**
IFN-g	-8.89	13	0.001**	-8.16	13	0.001**
IP-10	-7.88	13	0.001**	-8.04	13	0.001**
TNF-a	0.26	13	0.8	-0.35	13	0.73

Tear cytokine concentrations in pg/mL. BBA = bead-based assay. NS = non-stimulated tears. STIM = stimulated tears. TAB = Teknova assay buffer. DAB = Diaclone standard diluent buffer. t = t-test. Z = z-test. df = degrees of freedom.

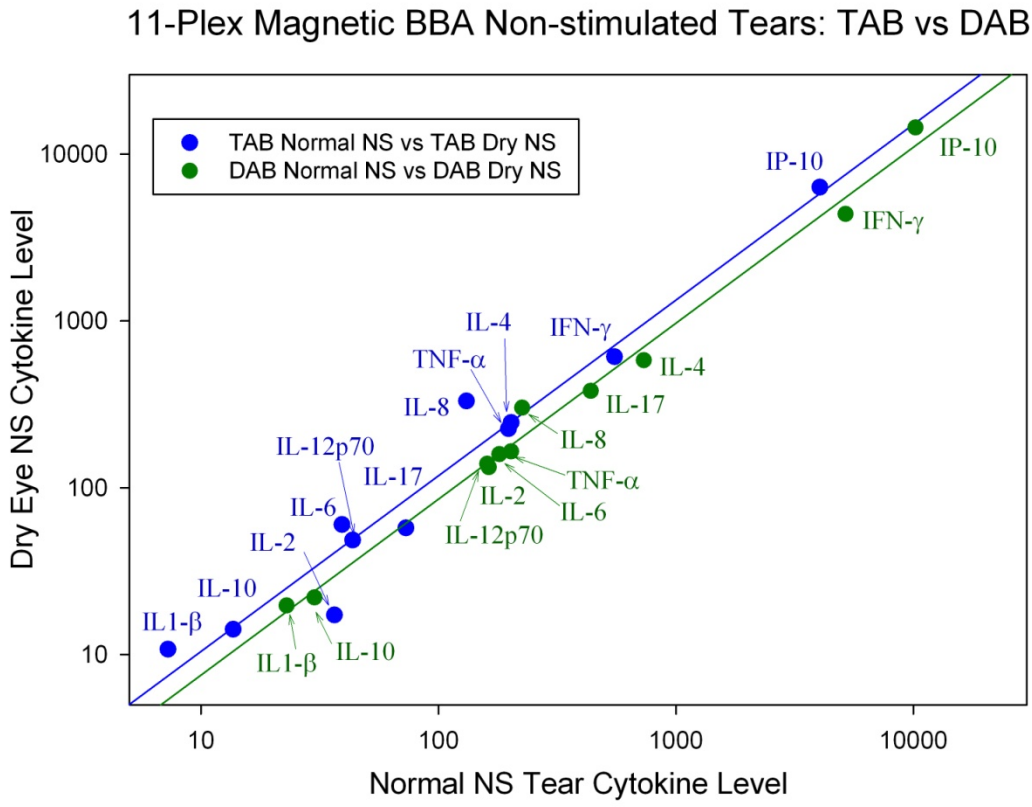

Figure 8a. 11-Plex Magnetic BBA Non-stimulated Tears: TAB vs. DAB. Rank ordering of cytokine levels is similar with both buffers despite DAB absolute levels being higher. Normal (n = 2, Subjects 1 and 2). Dry eye (n = 2, Subjects 21 and 24).

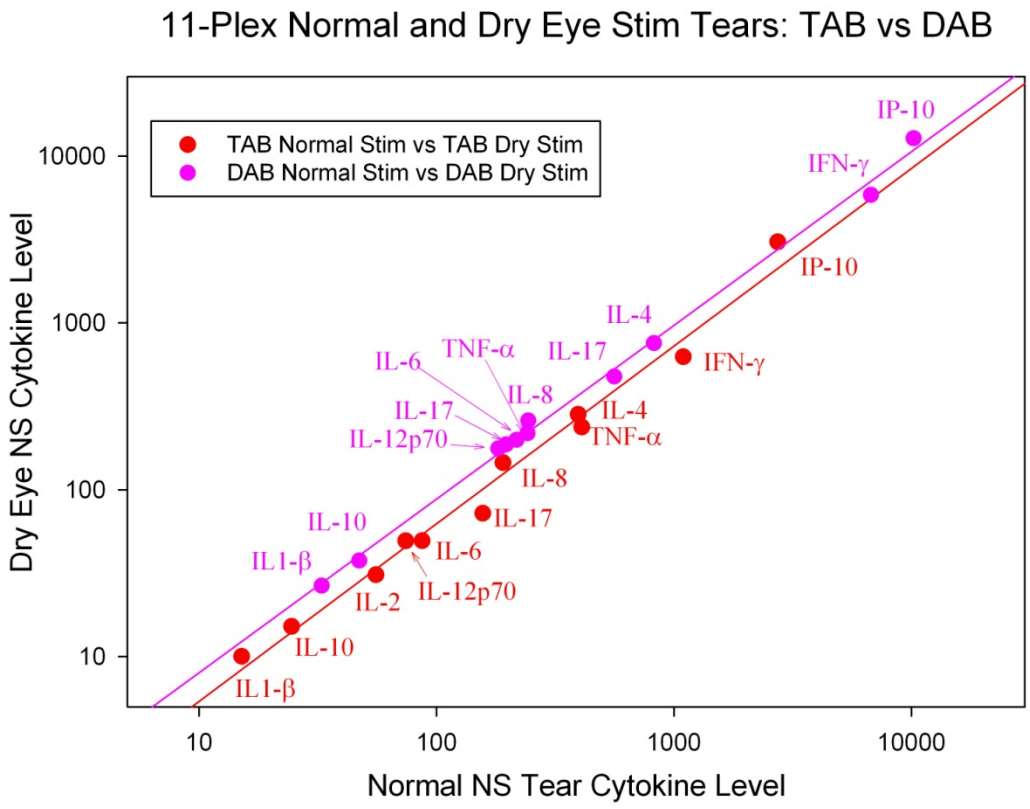

Figure 8b. 11-Plex Normal and Dry Eye Stimulated Tears: TAB vs. DAB. As with non-stimulated tears, rank ordering of cytokine levels is similar with both buffers and DAB absolute levels are consistently higher. TAB = Teknova assay buffer. DAB = Diaclone assay buffer. Normal (n = 2, Subjects 1 and 2). Dry eye (n = 2, Subjects 21 and 24).

Comparison to published cytokine concentrations. Cytokine levels in the 11-Plex magnetic BBA using TAB are closer to those reported by LaFrance et al [46] from this laboratory using a 27-Plex polystyrene bead-based assay (Table 22) than the current results for DAB. The only exceptions are IL-4 and IP-10. IL-4 is much higher with TAB than the published concentrations and less than DAB. IP-10 is much higher in LaFrance's report than both the current TAB and DAB cytokine concentrations [46].

Table 22

11-Plex Magnetic BBA and Published 27-Plex Polystyrene BBA (Non-stimulated Tears)

Cytokine	11-Plex TAB	SE	11-Plex DAB	SE	27-Plex PS BBA (NS) LaFrance [46]	SE
IL-1β	7.26	1.99	22.94	10.24	5.20	0.90
IL-2	36.48	4.08	162.88	55.58	61.90	9.20
IL-4	202.42	59.69	732.34	222.09	29.30	3.90
IL-6	39.24	10.35	180.36	65.09	35.20	5.50
IL-8	131.19	21.84	225.22	44.44	147.70	15.80
IL-10	13.66	3.68	30.03	24.03	23.60	4.90
IL-12p70	43.50	10.42	160.40	51.18	32.90	3.80
IL-17	72.96	30.12	437.64	150.16	72.20	12.70
IFN-γ	550.99	145.11	5,176.0	1,772.2	331.60	52.20
IP-10	4,036.2	630.41	10,203.1	2,461.4	23,622.3	3,500.6
TNF-α	197.03	66.94	202.11	71.81	175.90	74.60

Tear cytokine concentrations in pg/mL. BBA = bead-based assay. TAB = Teknova assay buffer. DAB = Diaclone standard diluent buffer. PS = polystyrene. SE = standard error. Normal (n = 2, Subjects 1 and 2).

TAB with Anti-Protease (Flowchart #9)

STIM samples were stored in TAB-AP and run on a Bio-Rad 27-Plex polystyrene BBA for Subject 21 (dry eye). Measured cytokine concentrations of tear samples stored in TAB-AP were less than tear samples stored in TAB without anti-protease, with the exception of IP-10 (Table 23). Paired t-tests showed that tear levels were significantly lower in AP buffer for the following cytokines: IL-1β, IL-2, IL-5, IL-6, IL-13, Eotaxin, GM-CSF, MIP-1α, RANTES, TNF-α, and VEGF. However, the rank ordering of cytokine concentration from high to low was very similar for the two buffer conditions. For 14 of the cytokines, rank order was the same; rank order differed by one for eight cytokines, and by two for three cytokines. The remaining two cytokines were undetected in both samples and could not be ranked.

Table 23

27-Plex Polystyrene BBA using Filter Plate: Comparison of TAB and TAB-AP

Cytokine	TAB	SE	TAB-AP	SE
IL-1β	4.01	0.33	3.30	0.20
IL-1ra	6,039.16	995.66	5,457.95	138.60
IL-2	71.08	7.78	57.78	2.59
IL-4	26.41	3.60	22.95	0.95
IL-5	14.67	1.86	10.43	0.22
IL-6	35.73	3.74	25.62	1.16
IL-7	126.65	15.35	114.60	12.64
IL-8	123.50	4.78	76.20	0.38
IL-9	28.62	0.85	10.33	7.10
IL-10	150.73	19.17	132.23	10.43
IL-12p70	47.37	1.16	45.75	1.39
IL-13	29.88	2.91	23.79	0.68
IL-15	4.58	0.33	3.70	0.26
IL-17	2.24	0.33	-	-
Eotaxin	102.81	2.04	53.14	6.25
FGF basic	-	-	-	-
G-CSF	19.49	2.37	14.35	1.40
GM-CSF	135.78	3.77	67.88	6.04
IFN-γ	431.12	65.35	351.55	7.13
IP-10	24,159.80	1,950.45	25,467.54	2,859.59
MCP-1	48.98	11.21	35.50	4.91
MIP-1α	96.67	9.41	59.32	0.96
MIP-1β	-	-	-	-
PDGF-BB	6.65	2.03	5.21	1.72
RANTES	180.74	12.56	154.16	7.47
TNF-α	328.29	4.42	232.84	2.23
VEGF	433.68	23.43	380.43	17.85

Tear cytokine concentrations in pg/mL. BBA = bead-based assay. TAB = Teknova assay buffer. TAB-AP = Teknova assay buffer with anti-protease. SE = standard error.

Cytokine Detection in Three Different Tear Sample Types using the 27-Plex Magnetic Bead-based Assay with TAB (Flowchart #10)

For the final sequence of assays, TAB was selected as the buffer for all samples and standard curves. A Bio-Rad 27-Plex magnetic BBA was used to determine TAB's ability to quantify cytokines in NS, WO, and STIM tear samples. Comparison of tear sample type was the project of another student in this laboratory. The focus in the current MS project was assay performance for the different sample types. In particular, the ability of the assay to detect cytokines in the WO tear sample collected one minute after the instillation of 10 μL sterile saline was of interest, because this represents a deliberate further dilution of the tear sample beyond the 1 in 10 used for assay. The sequence of tear collection for eight normal (Subjects 1, 2, 3, 4, 5, 6, 7, 8) and 6 dry eye subjects (Subjects 14, 15, 17, 18, 21, and 23) was as follows: one NS tear sample, two WO tear samples, and one STIM tear sample, which was divided into duplicate (Table 24). Four subjects (8, 14, 17, and 18) only collected a single WO tear sample and only one subject (17) had no STIM tear samples.

Sometimes the WO sample collected immediately after adding saline to the eye did not detect all 27 cytokines, but the second WO collected usually detected all 27 cytokines (Table 25). In addition, the concentrations of the second washout sample tended to be greater than the first washout sample. IL-9 was significantly higher in a dry eye subjects (Table 25) than in a normal subject (Table 26). This magnetic BBA showed varied levels of concentrations within a given subject unlike previous magnetic BBA studies run in this lab where cytokines would be either all low or all high within a given subject.

Table 24

27-Plex Magnetic BBA: 14 Subjects (Normal, n = 8 and Dry Eye, n =6)

Cytokine	NS	WO1	WO2	STIM
IL-1β	4.34	4.26	6.37	6.33
IL-1ra	1,023.08	879.34	815.02	673.10
IL-2	2.98	4.37	6.18	6.44
IL-4	13.92	12.40	17.61	17.97
IL-5	11.14	11.09	15.81	15.50
IL-6	23.52	21.84	32.40	30.89
IL-7	41.08	33.77	47.19	44.82
IL-8	99.19	64.79	80.75	69.34
IL-9	637.64	261.11	695.55	131.60
IL-9*	74.80	77.86	3.73	3.29
IL-10	9.73	8.67	12.75	12.39
IL-12(p70)	98.32	81.81	108.11	90.23
IL-13	12.06	10.29	14.32	13.26
IL-15	1.44	1.34	2.08	2.00
IL-17	8.34	7.56	13.50	15.15
Eotaxin	30.79	38.60	53.90	55.13
FGF basic	10.97	9.93	21.87	22.05
G-CSF	33.82	30.41	46.85	45.37
GM-CSF	-	-	-	-
IFN-γ	313.79	277.11	421.27	390.60
IP-10	3,714.84	2,939.99	3,158.80	2,630.94
MCP-1	9.39	7.91	12.79	10.84
MIP-1α	27.07	22.08	35.05	31.33
MIP-1β	20.89	15.87	21.32	18.59
PDGF-bb	21.98	23.52	39.56	44.34
RANTES	118.32	108.75	145.85	139.39
TNF-α	115.09	135.62	211.94	217.30
VEGF	137.46	99.96	134.46	124.72

Tear cytokine concentrations in pg/mL. *Indicates IL-9 concentration minus outlier. BBA = bead-based assay. NS = non-stimulated tears. WO = washout tears. STIM = stimulated tears. Buffer: TAB.

Table 25

27-Plex Magnetic BBA: Subject 15 (Dry Eye)

Cytokine	NS	WO1	WO2	STIM
IL-1β	5.37	1.73	5.61	8.54
IL-1ra	665.63	215.05	619.52	762.24
IL-2	1.69	-	2.48	5.99
IL-4	17.13	3.17	13.58	22.81
IL-5	13.12	3.32	11.96	18.11
IL-6	31.23	7.62	30.86	39.13
IL-7	39.43	13.9	35.22	51.86
IL-8	69.42	18.79	55.81	58.29
IL-9	7,954.50	2,643.37	5,896.46	366.96
IL-10	10.17	3.12	10.27	15.83
IL-12(p70)	111.87	44.34	91.18	92.82
IL-13	13.99	4.04	11.16	17.18
IL-15	1.81	0.27	1.51	2.36
IL-17	20.96	3.75	18.74	21.72
Eotaxin	39.92	-	28.35	68.34
FGF basic	8.25	-	6.32	18.49
G-CSF	38.83	9.45	45.40	54.81
GM-CSF	-	-	-	-
IFN-γ	409.74	73.24	358.63	480.55
IP-10	4,153.97	3,963.65	3,251.05	1,071.87
MCP-1	10.94	-	8.51	15.08
MIP-1α	48.28	17.22	44.83	39.00
MIP-1β	23.62	-	22.24	27.51
PDGF-bb	37.04	11.57	39.03	75.36
RANTES	135.75	52.01	142.98	171.28
TNF-α	113.66	-	157.23	219.8
VEGF	104.1	15.33	68.70	83.15

Tear cytokine concentrations in pg/mL. BBA = bead-based assay. NS = non-stimulated. WO = washout tears. STIM = stimulated tears. Buffer: TAB.

Table 26

27-Plex Magnetic BBA: Subject 3 (Normal)

Cytokine	NS	WO1	WO2	STIM
IL-1β	7.17	7.67	8.52	9.54
IL-1ra	992.84	789.42	867.35	965.31
IL-2	6.39	5.51	10.00	10.80
IL-4	19.21	20.36	23.28	22.13
IL-5	16.67	17.87	23.79	23.40
IL-6	30.86	33.1	41.41	43.32
IL-7	47.41	47.58	65.34	60.31
IL-8	117.71	63.01	83.78	87.47
IL-9	129.67	151.73	1.71	1.94
IL-10	12.00	12.66	17.58	1.68
IL-12(p70)	113.44	106.95	140.31	113.44
IL-13	15.89	15.54	17.46	16.44
IL-15	2.52	2.30	3.18	3.07
IL-17	16.15	14.66	17.65	20.68
Eotaxin	42.84	50.94	61.57	74.34
FGF basic	-	7.52	22.85	21.05
G-CSF	44.88	56.89	63.12	60.66
GM-CSF	-	-	-	-
IFN-γ	394.21	403.09	500.40	531.25
IP-10	1,674.60	1,368.50	1,327.90	1,272.10
MCP-1	11.24	9.77	16.83	13.95
MIP-1α	35.82	37.58	41.50	46.25
MIP-1β	31.68	22.66	29.48	28.37
PDGF-bb	39.03	50.56	72.46	57.16
RANTES	145.36	146.54	175.74	200.7
TNF-α	185.03	262.73	336.6	317.01
VEGF	112.28	118.48	151.9	154.06

Tear cytokine concentrations in pg/mL. BBA = bead-based assay. NS = non-stimulated tears. WO = washout tears. STIM = stimulated tears. Buffer: TAB.

Table 27 shows that the previously reported polystyrene BBA results [35] showed greater concentrations for most cytokines when compared to the current magnetic BBA. Both BBAs detected all cytokines except for GM-CSF, which was below the limit of detection in this magnetic BBA. The distributions for all cytokines were normal except for TNF-α. The Mann-Whitney U test, which is a non-parametric test used to determine if a difference exists between two groups, showed there was a significant difference between this magnetic BBA and the published polystyrene BBA results for NS tears. Therefore, the magnetic BBA does not appear to be equivalent to the polystyrene BBA.

Table 27a

27-Plex Magnetic BBA: Eight Subjects (Normal)

Cytokine	NS	n	SD	WO1	WO2	STIM	27-Plex PS BBA (NS) LaFrance[46]	N	SD	Mann-Whitney U (or t)	df (t)	P value
IL-1β	4.26	8	1.99	4.82	6.24	6.53	5.2	20	3.94	42	-	0.188
IL-1ra	1,001.75	8	675.44	899.67	794.92	644.02	9,589.40	31	7,964.90	15	-	0.001
IL-2	4.16	3	2.94	4.47	8.95	9.39	61.9	29	48.72	7	-	0.024
IL-4	12.96	8	4.10	13.31	16.92	17.32	29.3	32	21.72	55	-	0.032
IL-5	11.34	8	4.07	12.48	15.74	15.75	13.7	31	16.44	78	-	0.246
IL-6	22.4	8	6.83	24.18	31.68	31.31	35.2	31	30.13	95	-	0.449
IL-7	41.22	8	11.03	37.44	49.08	47.47	382.3	31	176.97	0	-	0.001
IL-8	91.01	8	40.53	68.05	81.14	75.10	147.7	30	85.13	93	-	0.038
IL-9	73.21	8	37.33	84.13	115.25	113.04	35.6	20	21.43	70	-	0.412
IL-10	9.14	8	2.94	9.03	12.91	12.32	23.6	26	24.53	55	-	0.099
IL-12(p70)	94.91	8	12.45	84.70	108.78	93.62	32.9	24	18.26	33	-	0.005
IL-13	11.84	8	2.96	11.24	14.69	13.35	19.6	27	12.25	71	-	0.123
IL-15	0.31	1	-	0.11	0.96	1.13	14	2	1.78	-	-	-
IL-17	7.18	6	5.63	10.28	14.04	14.13	72.2	20	55.55	4	-	0.001

Tear cytokine concentrations in pg/mL. BBA = bead-based assay. NS = non-stimulated tears. n = number of subjects. SD = standard deviation. WO = washout tears. STIM = stimulated tears. PS = polystyrene. df = degrees of freedom. Buffer: TAB.

Table 27b

27-Plex Magnetic BBA: Eight Subjects (Normal)

Cytokine	NS	n	SD	WO1	WO2	STIM	27-Plex PS BBA (NS) LaFrance[46]	N	SD	Mann-Whitney U (or t)	df (t)	p
Eotaxin	34.4	5	12.46	36.39	56.09	59.12	293.7	28	157.59	0	-	0.001
FGF basic	2.25	3	1.56	7.56	8.85	13.03	-		-	-	-	-
G-CSF	31.03	8	12.14	33.95	46.12	46.57	43.3	29	23.30	70	-	0.093
GM-CSF	OOR <	0	-	OOR <	OOR <	OOR <	95.7	10	66.83	-	-	-
IFN-γ	292.94	8	93.35	304.71	412.38	384.8	331.6	30	281.25	115	-	0.985
IP-10	3,933.36	8	2,468.17	3,280.29	3,403.08	3,507.77	23,622.30	30	18,861.06	8	-	0.001
MCP-1	6.95	8	4.03	7.76	12.16	10.23	132.5	22	93.27	7	-	0.001
MIP-1α	24.35	8	8.27	26.00	33.01	32.24	26.2	29	17.47	99	-	0.983
MIP-1β	15.72	8	11.36	14.94	20.16	20.96	48.5	27	52.07	38	-	0.016
PDGF-bb	19.4	8	10.30	25.79	37.12	40.96	31.6	29	23.30	83	-	0.23
RANTES	120.18	8	26.06	119.09	145.75	144.78	36.1	28	20.28	11	-	0.001
TNF-α	93.06	8	74.92	123.46	202.10	189.92	175.9	4	128.35	1.314	10	0.218
VEGF	123.04	8	47.29	102.46	133.49	141.56	2,608.50	32	1,391.05	2	-	0.001

Tear cytokine concentrations in pg/mL. BBA = bead-based assay. NS = non-stimulated tears. n = number of subjects. SD = standard deviation. WO = washout tears. STIM = stimulated tears. PS = polystyrene. df = degrees of freedom. Buffer: TAB.

Comparison of Polystyrene with Magnetic BBA (Flowchart #11 & 12)

For the final comparisons of polystyrene and magnetic BBAs, the optimal assay modifications and buffer (automatic wash system, filter plate, and TAB with anti-protease) were used. The overall cytokine profile and individual cytokine levels for each assay type were also determined. A greater number of both normal and dry eye subjects were in the final assay comparisons than in earlier assays in this study.

Linearity of Dilution and Spike-recovery (Flowchart #11)

Linearity of dilution and spike-recovery were compared on two separate plates for Bio-Rad 27-Plex polystyrene and magnetic BBAs (filter plates). The pooled tear samples of Subject 1 (normal) were run on both plates. Although the results for TAB-AP were not significantly better than TAB, anti-protease tablets were added to TAB as a precaution to prevent any potential protein breakdown in the tears.

Linearity of dilution recovery was much better in the polystyrene BBA when compared to the magnetic BBA (Table 28). The polystyrene BBA showed good recovery down to 2 µL for 12 cytokines and down to 1 µL for four cytokines. On the other hand, the magnetic BBA was only able to detect four cytokines at the 4 µL level and none at the lower tear volumes. In addition, the polystyrene BBA detected all cytokines except two (FGF-basic and MIP-1β), while the magnetic BBA was unable to detect 11 of the 27 cytokines at the 5 uL level. The polystyrene BBA appeared to be superior to the magnetic BBA in all aspects of linearity of dilution.

Table 28a

27-Plex Polystyrene and Magnetic BBAs: Linearity of Dilution (TAB-AP)

	IL-1β	IL-1ra	IL-2	IL-4	IL-5	IL-6	IL-7	IL-8	IL-9	IL-10	IL-12p70
						Polystyrene Bead Plate					
5 µL level	1.13	132.86	32.27	11.63	5.23	16.35	79.58	92.85	24.17	85.63	38.01
4 µL Rec.	133.6%	94.5%	105.2%	109.1%	82.6%	95.2%	109.9%	94.9%	86.7%*	112.1%	118.9%
3 µL Rec.	137.2%	86.1%	95.2%*	98.7%	84.7%	84.5%*	102.5%	94.5%	56.4%	104.9%*	116.7%*
2 µL Rec.	206.2%	84.1%	67.9%	80.0%*	74.6%*	62.0%	129.5%*	99.1%	61.6%	161.3%	160.6%
1 µL Rec.	390.3%	45.1%	-	37.3%	85.5%	28.4%	153.0%	104.4%*	126.1%*	232.9%	174.2%
						Magnetic Bead Plate					
5 µL level	1.33	90.64	-	3.53	3.23	6.37	19.27	24.49	-	4.32	57.48
4 µL Rec.	90.2%*	86.2%*	-	86.7%*	87.9%*	95.0%*	79.3%*	81.4%*	-	69.7%	71.9%*
3 µL Rec.	58.6%	50.0%	-	30.6%	61.9%	32.8%	53.7%	51.5%	-	42.1%	49.7%
2 µL Rec.	46.6%	25.2%	-	12.7%	50.2%	-	50.0%	45.4%	-	45.6%	57.7%
1 µL Rec.	34.6%	4.3%	-	-	37.5%	-	29.9%	13.8%	-	26.4%	35.6%

5 µL level concentrations in pg/mL. BBA = bead-based assay. TAB-AP = Teknova assay buffer with anti-protease. Rec. = recovery.
*Indicates lowest volume at which linearity of dilution recovery was within 30% of the 5 µL value. STIM tears.

Table 28b

27-Plex Polystyrene and Magnetic BBAs: Linearity of Dilution (TAB-AP)

	IL-13	IL-15	IL-17	Eotaxin	FGF-basic	G-CSF	GM-CSF	IFN-γ	IP-10	MCP-1	MIP-1α
					Polystyrene Bead Plate						
5 µL level	11.00	1.92	4.56	53.80	-	5.43	52.75	270.29	13,001	19.03	34.97
4 µL Rec.	117.0%	110.9%	95.4%*	83.0%*	-	104.6%	88.5%*	106.3%	120.4%	114.8%	67.1%
3 µL Rec.	113.0%*	79.2%	42.3%	17.4%	-	104.2%	63.5%	85.5%*	105.9%	90.6%	-
2 µL Rec.	160.4%	90.1%*	122.4%	-	-	123.6%*	52.2%	64.1%	115.1%	114.2%*	-
1 µL Rec.	218.7%	131.3%	172.1%	-	-	141.8%	9.9%	28.3%	128.6%*	130.8%	-
					Magnetic Bead Plate						
5 µL level	10.45	-	-	-	-	6.49	-	24.95	1,595.5	-	-
4 µL Rec.	88.1%*	-	-	-	-	93.7%*	-	71.3%*	75.0%*	-	-
3 µL Rec.	57.3%	-	-	-	-	15.1%	-	-	49.8%	-	-
2 µL Rec.	58.3%	-	-	-	-	-	-	-	61.3%	-	-
1 µL Rec.	43.3%	-	-	-	-	-	-	-	38.1%	-	-

5 µL level concentrations in pg/mL. BBA = bead-based assay. TAB-AP = Teknova assay buffer with anti-protease. Rec. = recovery.
*Indicates lowest volume at which linearity of dilution recovery was within 30% of the 5 µL value. STIM tears.

Table 28c

27-Plex Polystyrene and Magnetic BBAs: Linearity of Dilution (TAB-AP)

	MIP-1β	PDGF-bb	RANTES	TNF-a	VEGF
		Polystyrene Bead Plate			
5 µL level	-	18.14	91.67	127.60	370.68
4 µL Rec.	-	113.0%	110.8%	100.5%	115.7%
3 µL Rec.	-	89.4%	105.0%	79.9%*	91.7%
2 µL Rec.	-	129.4%*	96.6%*	52.8%	116.7%
1 µL Rec.	-	162.1%	46.8%	11.7%	125.7%*
		Magnetic Bead Plate			
5 µL level	-	3.56	24.8	-	44.19
4 µL Rec.	-	41.9%	72.3%	-	58.6%
3 µL Rec.	-	-	-	-	21.2%
2 µL Rec.	-	-	-	-	24.5%
1 µL Rec.	-	-	-	-	-

5 µL level concentrations in pg/mL. BBA = bead-based assay. TAB-AP = Teknova assay buffer with anti-protease. Rec. = recovery.
*Indicates lowest volume at which linearity of dilution recovery was within 30% of the 5 µL value. STIM tears.

Tear samples for Subject 1 (normal) were spiked with standards at high (Standard 7), medium (Standard 5), and low (Standard 3) levels and recovery was noted (Table 29). Spiked samples were run on both the 27-Plex polystyrene and magnetic BBAs. The polystyrene BBA did not detect FGF-basic and MIP-1β. The magnetic BBA did not detect these two cytokines in addition to nine other cytokines. For both plates, recovery was closest to 100% for the low spikes and progressively decreased for the medium and high spikes. Recovery was considerably lower overall for the magnetic BBA relative to the polystyrene BBA. Subject 1 elicited very low levels of many tear cytokines. This appeared to be a major contributor to the poor spike-recovery results, in particular for the magnetic BBA.

Table 29a

27-Plex Polystyrene and Magnetic BBAs: Spike-recovery (Subject 1)

	IL-1b	IL-1ra	IL-2	IL-4	IL-5	IL-6	IL-7	IL-8	IL-9	IL-10	IL-12p70
Recovery						Polystyrene Bead Plate					
High Spike	14.6%	53.1%	5.5%	12.8%	17.2%	37.1%	23.4%	33.4%	34.6%	34.7%	29.7%
Medium Spike	63.1%	77.0%	10.9%	28.4%	80.9%	45.3%	41.4%	49.6%	54.4%	52.2%	51.3%
Low Spike	140.6%	148.4%	66.4%	97.8%	203.5%	163.5%	102.7%	108.5%	119.3%	126.9%	130.3%
Recovery						Magnetic Bead Plate					
High Spike	3.7%	2.8%	-	5.9%	2.9%	2.8%	5.0%	6.6%	-	6.9%	5.1%
Medium Spike	4.7%	11.5%	-	12.2%	4.0%	3.7%	6.1%	9.6%	-	9.3%	10.3%
Low Spike	6.6%	56.8%	-	33.5%	8.8%	12.0%	24.0%	37.0%	-	17.0%	38.2%

BBA = bead-based assay. Buffer: Teknova assay buffer with anti-protease (TAB-AP).

Table 29b

27-Plex Polystyrene and Magnetic BBAs: Spike-recovery (Subject 1)

	IL-13	IL-15	IL-17	Eotaxin	FGF-basic	G-CSF	GM-CSF	IFN-g	IP-10	MCP-1	MIP-1a
Recovery					Polystyrene Bead Plate						
High Spike	40.4%	29.1%	47.9%	17.8%	-	20.2%	24.3%	9.4%	64.2%	52.5%	-
Medium Spike	84.1%	66.2%	75.8%	46.7%	-	39.7%	34.6%	36.5%	88.3%	74.1%	49.7%
Low Spike	222.1%	148.0%	174.1%	112.3%	-	128.4%	102.2%	98.0%	84.2%	173.0%	153.3%
Recovery					Magnetic Bead Plate						
High Spike	7.5%	-	-	-	-	41.6%	-	24.8%	123.5%	-	-
Medium Spike	11.1%	-	-	-	-	41.9%	-	80.3%	418.3%	-	-
Low Spike	21.3%	-	-	-	-	122.5%	-	468.6%	696.3%	-	-

BBA = bead-based assay. Buffer: Teknova assay buffer with anti-protease (TAB-AP).

Table 29c

27-Plex Polystyrene and Magnetic BBAs: Spike-recovery (Subject 1)

	MIP-1b	PDGF-bb	RANTES	TNF-a	VEGF
Recovery			Polystyrene Bead Plate		
High Spike	-	40.6%	13.2%	37.3%	24.0%
Medium Spike	-	57.7%	26.0%	55.8%	75.2%
Low Spike	-	135.3%	99.3%	137.6%	96.9%
Recovery			Magnetic Bead Plate		
High Spike	-	6.7%	62.4%	-	40.4%
Medium Spike	-	7.6%	124.7%	-	90.3%
Low Spike	-	11.0%	411.1%	-	330.1%

BBA = bead-based assay. Buffer: Teknova assay buffer with anti-protease (TAB-AP).

The limits of quantification were very similar for the polystyrene and magnetic BBAs (Table 30). The polystyrene BBA had a LLOQ less than the magnetic BBA for 13 of 27 cytokines. The magnetic BBA had an ULOQ greater than the polystyrene BBA for 17 cytokines.

Most of the high and medium spike recoveries for the magnetic BBA were very poor in the previous study and it was determined that the low tear cytokine levels for Subject 1 were a major contributing factor. Therefore, a second study of linearity of dilution and spike-recovery on polystyrene and magnetic BBAs using two subjects who had previously shown higher tear cytokine levels. On the polystyrene plate, NS and STIM tears were run for Subject 23 (dry). On the magnetic plate, NS tears from Subject 23 and STIM tears from Subjects 15 (dry) and 23 were run. The magnetic BBA produced cytokine concentrations greater than the polystyrene BBA for 15 of 27 cytokine for NS tears and 18 of 27 cytokines for STIM tears (Table 31).

Table 30

Limits of Quantification: 27-Plex Polystyrene and Magnetic BBAs

	Polystyrene		Magnetic	
Cytokine	LLOQ	ULOQ	LLOQ	ULOQ
IL-1β	0.62	215.71	0.63	2,846.90
IL-1ra	0.90	15,325.17	0.95	14,291.31
IL-2	0.33	6,365.65	0.32	4,295.11
IL-4	0.07	273.12	0.26	1,078.92
IL-5	0.59	216.09	0.59	806.57
IL-6	0.50	7,042.75	0.81	12,214.72
IL-7	0.57	188.78	0.54	702.39
IL-8	0.50	8,148.54	0.48	7,256.35
IL-9	0.71	2,184.66	0.56	8,587.61
IL-10	0.52	7,421.24	0.49	606.88
IL-12p70	0.73	9,070.75	0.74	9,504.20
IL-13	0.69	253.57	0.73	1,046.22
IL-15	0.58	204.43	0.57	798.04
IL-17	0.49	1,607.29	0.46	6,001.40
Eotaxin	5.90	176.06	6.11	8,209.16
FGF basic	8.48	874.42	7.26	3,508.65
G-CSF	0.60	199.09	0.59	7,238.15
GM-CSF	0.29	4,360.69	3.65	4,042.39
IFN-γ	0.35	7,962.57	5.43	7,252.34
IP-10	0.75	8,807.31	0.61	11,317.53
MCP-1	0.40	1,901.53	0.32	6,474.18
MIP-1α	0.84	120.35	0.92	456.40
MIP-1β	9.27	408.96	1.00	349.55
PDGF-bb	0.46	7,396.58	0.45	7,034.92
RANTES	1.54	663.69	1.73	5,043.39
TNF-α	1.53	26,879.66	6.06	22,803.77
VEGF	0.63	10,585.53	0.56	9,635.70

Tear cytokine concentrations in pg/mL. BBA = bead-based assay. LLOQ = lower limit of quantification. ULOQ = upper limit of quantification. Buffer: Teknova assay buffer with anti-protease (TAB-AP).

Table 31

Non-stimulated and Stimulated Levels for 27-Plex Polystyrene and Magnetic BBAs

	Non-Stimulated		Stimulated	
	Polystyrene	Magnetic	Polystyrene	Magnetic
IL-1β	4.15	29.43	11.26	43.87
IL-1ra	7,884.60	2,024.98	1,058.94	2,569.94
IL-2	125.53	69.91	135.51	101.65
IL-4	49.66	45.37	56.81	59.84
IL-5	13.97	54.01	44.58	79.86
IL-6	68.80	89.98	104.07	128.48
IL-7	112.57	77.56	109.00	101.86
IL-8	1,077.20	694.35	211.58	733.71
IL-9	91.69	826.73	189.47	1,099.76
IL-10	204.22	28.03	197.17	42.78
IL-12p70	63.78	109.69	129.55	152.46
IL-13	20.67	37.97	46.33	55.33
IL-15	6.29	12.73	8.10	22.76
IL-17	18.28	89.68	36.34	129.67
Eotaxin	227.78	153.14	346.60	211.46
FGF basic	-	325.73	13.76	483.59
G-CSF	50.16	67.45	85.33	99.70
GM-CSF	257.26	252.33	769.61	362.77
IFN-γ	1,204.65	903.25	1,405.53	1,240.10
IP-10	31,734.39	2,376.53	10,952.50	1,603.43
MCP-1	32.04	116.40	15.73	154.57
MIP-1α	136.36	86.40	250.30	114.06
MIP-1β	-	83.11	-	89.64
PDGF-bb	14.53	55.59	24.60	114.54
RANTES	296.52	311.35	309.79	376.09
TNF-α	1,072.86	2,461.39	2,120.06	3,617.42
VEGF	739.44	152.50	468.32	161.48

Tear cytokine concentrations in pg/mL. BBA = bead-based assay. Buffer: Teknova assay buffer with anti-protease (TAB-AP).

Table 32 shows linearity of dilution for the NS tear samples of Subject 23 and Table 33 shows linearity of dilution for the STIM tear samples of Subjects 15 and 23. Each table compares a 27-Plex polystyrene BBA to a 27-Plex magnetic BBA. Neither plate type showed superior linearity of dilution for NS tear samples (Table 32). For STIM tears, Subject 15's tears on the polystyrene and magnetic plates showed varied results for linearity of dilution (Table 33). For Subject 23 on the magnetic plate, tear samples exceeding the standard 5 uL volume were run to determine if the BBA would be more reliable when larger tear volumes are used. For larger volumes, there was a clear departure from linear increases in cytokine concentration. For most cytokines, the 25 uL volume produced substantial underestimation of tear cytokine levels. However, recovery was within acceptable limits down to 2 to 3 µL for most cytokines. This indicates that increased tear volumes are not a viable solution.

Table 32a

Linearity of Dilution for Non-stimulated Tear Samples on Polystyrene and Magnetic BBAs

		IL-1β	IL-1ra	IL-2	IL-4	IL-5	IL-6	IL-7	IL-8	IL-9	IL-10
						Polystyrene Bead Plate					
Subject 23	5 µL level	4.2	7,884.6	125.5	49.7	14.0	68.8	112.6	1,077.2	91.7	204.2
	3.66 µL Rec.	88.4%	89.6%	76.4%*	74.6%*	54.7%	69.1%	86.5%	95.0%	69.9%	117.9%*
	2.33 µL Rec.	107.0%*	94.3%	58.4%	66.1%	16.5%	54.7%	95.6%	101.5%	57.0%	151.1%
	1 µL Rec.	135.2%	102.4%*	32.5%	42.3%	-	25.8%	118.0%*	108.5%*	14.4%	218.1%
						Magnetic Bead Plate					
Subject 23	5 µL level	23.5	1,970.6	63.8	41.1	46.7	79.7	70.0	795.7	483.6	23.1
	3.66 µL Rec.	96.1%	104.4%	83.9%*	105.2%	100.5%	97.9%	107.8%	110.3%	98.4%	97.8%
	2.33 µL Rec.	104.6%*	119.2%	66.9%	114.6%*	101.7%*	105.1%*	120.2%	112.8%	98.2%*	94.2%*
	1 µL Rec.	0.5%	97.2%*	-	63.1%	47.2%	42.5%	74.6%*	96.6%*	20.5%	-

5 µL level concentrations in pg/mL. BBA = bead-based assay. Rec. = recovery. *Indicates lowest volume at which linearity of dilution recovery was within 30% of the 5 µL value. Buffer: Teknova assay buffer with anti-protease (TAB-AP).

Table 32b

Linearity of Dilution for Non-stimulated Tear Samples on Polystyrene and Magnetic BBAs

		IL-12p70	IL-13	IL-15	IL-17	Eotaxin	FGF-basic	G-CSF	GM-CSF	IFN-γ	IP-10
						Polystyrene Bead Plate					
Subject 23	5 µL level	63.8	20.7	6.3	18.3	227.8	-	50.2	257.3	1,204.7	3,1734.4
	3.66 µL Rec.	106.8%	101.6%*	70.1%*	68.8%	63.7%	-	92.5%	49.0%	75.0%*	105.2%*
	2.33 µL Rec.	118.9%*	144.3%	34.5%	45.8%	26.5%	-	107.0%	28.7%	53.3%	170.6%
	1 µL Rec.	160.0%	205.1%	-	27.4%	-	-	128.7%*	8.8%	30.8%	176.3%
						Magnetic Bead Plate					
Subject 23	5 µL level	97.2	32.1	10.4	78.4	121.4	245.6	56.8	222.2	796.8	2,803.7
	3.66 µL Rec.	110.5%	119.4%	77.3%*	94.3%	106.9%	68.6%	98.7%	87.1%	105.2%	143.8%
	2.33 µL Rec.	115.2%	121.3%*	21.1%	85.0%*	128.6%*	-	106.2%*	96.0%*	102.6%*	197.2%
	1 µL Rec.	94.9%*	53.3%	-	-	35.2%	-	46.1%	-	52.4%	450.7%

5 µL level concentrations in pg/mL. BBA = bead-based assay. Rec. = recovery. *Indicates lowest volume at which linearity of dilution recovery was within 30% of the 5 µL value. Buffer: Teknova assay buffer with anti-protease (TAB-AP).

Table 32c

Linearity of Dilution for Non-stimulated Tear Samples on Polystyrene and Magnetic BBAs

		MCP-1	MIP-1α	MIP-1β	PDGF-bb	RANTES	TNF-α	VEGF
					Polystyrene Bead Plate			
Subject 23	5 µL level	32.0	136.4	-	14.5	296.5	1,072.9	739.4
	3.66 µL Rec.	68.6%	66.2%	-	78.8%	87.8%	65.9%	92.4%
	2.33 µL Rec.	64.0%	-	-	104.2%	83.2%	50.9%	107.7%
	1 µL Rec.	2.0%	-	-	110.1%*	72.3%*	-	91.4%*
					Magnetic Bead Plate			
Subject 23	5 µL level	101.8	73.2	89.8	22.9	294.7	2,110.4	174.3
	3.66 µL Rec.	110.9%	111.8%	122.7%*	103.7%	112.2%*	111.1%	113.1%
	2.33 µL Rec.	102.3%*	121.2%*	136.9%	94.3%*	134.5%	103.5%*	106.6%
	1 µL Rec.	-	-	128.5%	2.9%	118.8%	-	93.0%*

5 µL level concentrations in pg/mL. BBA = bead-based assay. Rec. = recovery. *Indicates lowest volume at which linearity of dilution recovery was within 30% of the 5 µL value. Buffer: Teknova assay buffer with anti-protease (TAB-AP).

Table 33a

Linearity of Dilution for Stimulated Tear Samples on Polystyrene and Magnetic BBAs

		IL-1β	IL-1ra	IL-2	IL-4	IL-5	IL-6	IL-7	IL-8	IL-9	IL-10
						Polystyrene Bead Plate					
Subject 23	5 µL level	11.3	1,058.9	135.5	56.8	44.6	104.1	109.0	211.6	189.5	197.2
	3.66 µL Rec.	83.4%	76.3%*	133.5%	125.8%*	86.0%	91.9%	90.2%	89.6%	68.3%	108.9%
	2.33 µL Rec.	80.6%	57.1%	151.8%	153.7%	91.2%*	88.0%	87.8%	82.7%*	49.1%	126.3%*
	1 µL Rec.	85.6%*	25.2%	160.0%	162.0%	59.2%	72.5%*	82.9%*	49.9%	18.5%	151.3%
						Magnetic Bead Plate					
Subject 23	5 µL level	52.3	3,060.6	127.3	70.1	98.4	156.7	118.6	874.4	1,029.7	52.6
	3.66 µL Rec.	125.6%	121.7%	117.0%*	123.0%	122.4%	126.3%	128.9%	129.4%	117.6%*	119.8%*
	2.33 µL Rec.	71.4%*	87.3%	63.3%	87.3%	80.5%*	77.6%*	101.2%	82.6%	65.5%	61.2%
	1 µL Rec.	54.8%	76.5%*	-	96.5%*	66.7%	62.9%	92.8%*	72.6%*	46.3%	26.9%
Subject 15	25 µL Rec.	31.2%	33.3%	35.4%	31.6%	36.9%	38.1%	34.7%	34.2%	29.1%	37.2%
	14.62 µL Rec.	42.4%	44.2%	39.6%	38.4%	46.8%	44.0%	48.1%	47.8%	37.1%	50.1%
	8.56 µL Rec.	70.1%*	75.0%*	77.0%*	79.4%*	76.4%*	86.0%*	73.6%*	74.9%*	64.4%	80.0%*
	5 µL level	35.4	2,079.3	76.0	49.6	61.4	100.2	85.2	593.0	1,169.8	33.0
	4 µL Rec.	107.1%	113.6%	106.4%	107.8%	109.1%	116.2%	114.2%	110.3%	113.3%	105.9%
	3 µL Rec.	103.3%	110.7%	93.8%	109.2%	108.9%	108.1%	111.3%	101.2%	123.0%*	90.3%
	2 µL Rec.	105.9%*	111.5%*	81.6%*	125.8%*	109.8%*	119.2%*	126.3%*	110.1%*	136.9%	86.5%*
	1 µL Rec.	15.2%	41.7%	-	59.1%	37.0%	37.0%	56.8%	44.4%	103.2%	-

5 µL level concentrations in pg/mL. BBA = bead-based assay. Rec. = recovery. *Indicates lowest and greatest volume at which linearity of dilution recovery was within 30% of the 5 µL value. Buffer: Teknova assay buffer with anti-protease (TAB-AP).

Table 33b

Linearity of Dilution for Stimulated Tear Samples on Polystyrene and Magnetic BBAs

		IL-12p70	IL-13	IL-15	IL-17	Eotaxin	FGF-basic	G-CSF	GM-CSF	IFN-γ	IP-10
						Polystyrene Bead Plate					
Subject 23	5 µL level	129.6	46.3	8.1	36.3	346.6	13.8	85.3	769.6	1,405.5	10,952.5
	3.66 µL Rec.	98.3%	85.2%	56.7%	83.9%*	88.2%	-	90.8%	82.2%	128.1%*	126.9%*
	2.33 µL Rec.	96.8%	89.2%	17.9%	46.5%	77.5%*	-	96.1%	70.0%*	155.8%	162.2%
	1 µL Rec.	92.0%*	77.7%*	-	37.6%	13.6%	-	98.3%*	43.5%	156.2%	220.7%
						Magnetic Bead Plate					
Subject 23	5 µL level	182.7	66.8	30.5	158.3	238.1	561.4	121.3	443.1	1,470.5	1,257.5
	3.66 µL Rec.	116.5%	119.9%	113.6%*	122.5%	126.1%	128.0%*	126.9%	124.8%	115.6%	129.3%*
	2.33 µL Rec.	82.3%	79.9%*	37.1%	74.1%*	79.7%*	65.4%	71.1%*	71.3%*	84.7%	187.8%
	1 µL Rec.	82.3%*	65.3%	-	34.4%	65.3%	-	57.3%	38.4%	71.0%*	306.9%
Subject 15	25 µL Rec.	39.0%	33.3%	46.9%	35.7%	32.2%	33.6%	33.0%	34.6%	31.5%	15.7%
	14.62 µL Rec.	40.7%	42.7%	51.1%	45.3%	46.9%	40.7%	46.5%	43.7%	50.8%	21.8%
	8.56 µL Rec.	74.4%*	78.9%*	77.3%*	78.3%*	79.0%*	76.1%*	79.7%*	76.2%*	72.5%*	35.5%
	5 µL level	122.2	43.9	15.0	101.0	184.9	405.8	78.1	282.4	1,009.7	1,949.4
	4 µL Rec.	115.3%	111.1%	108.1%	111.1%	105.8%	110.6%	110.5%	110.4%	109.6%	126.4%*
	3 µL Rec.	111.2%	108.8%	79.4%*	106.0%	100.5%	98.5%*	99.1%	98.6%	112.6%	209.3%
	2 µL Rec.	124.9%*	120.7%*	51.8%	103.9%*	103.2%*	59.6%	104.0%*	97.9%*	122.8%*	346.8%
	1 µL Rec.	59.1%	16.0%	-	-	24.1%	-	27.7%	-	41.3%	566.1%

5 µL level concentrations in pg/mL. BBA = bead-based assay. Rec. = recovery. *Indicates lowest and greatest volume at which linearity of dilution recovery was within 30% of the 5 µL value. Buffer: Teknova assay buffer with anti-protease (TAB-AP).

Table 33c

Linearity of Dilution for Stimulated Tear Samples on Polystyrene and Magnetic BBAs

		MCP-1	MIP-1α	MIP-1β	PDGF-bb	RANTES	TNF-α	VEGF
					Polystyrene Bead Plate			
Subject 23	5 µL level	15.7	250.3	-	24.6	309.8	2,120.1	468.3
	3.66 µL Rec.	56.5%	99.0%	-	87.4%	130.6%	89.3%	116.7%
	2.33 µL Rec.	19.6%	111.8%*	-	72.3%*	171.9%	85.9%*	119.3%
	1 µL Rec.	-	60.5%	-	54.3%	203.7%	55.2%	127.3%*
					Magnetic Bead Plate			
Subject 23	5 µL level	178.1	128.5	102.8	140.8	424.2	4,422.5	192.2
	3.66 µL Rec.	129.1%	133.5%	125.7%	121.2%*	127.8%	118.5%	130.3%
	2.33 µL Rec.	90.7%*	100.5%*	99.1%	59.9%	120.8%*	79.3%*	64.7%
	1 µL Rec.	61.7%	46.6%	98.0%*	41.8%	140.2%	49.7%	-
Subject 15	25 µL Rec.	33.8%	29.0%	30.3%	41.1%	29.6%	35.2%	46.1%
	14.62 µL Rec.	42.0%	46.8%	40.0%	51.0%	40.9%	47.2%	51.8%
	8.56 µL Rec.	63.4%*	71.6%*	69.9%	88.0%*	69.4%	72.6%*	74.7%*
	5 µL level	131.0	99.6	76.5	88.3	328.0	2,812.3	130.7
	4 µL Rec.	115.3%	112.8%	114.6%	98.5%	118.3%	113.9%	109.5%
	3 µL Rec.	104.4%	120.2%*	118.5%*	88.1%	124.8%*	109.5%	96.3%
	2 µL Rec.	120.0%*	135.8%	137.9%	83.6%*	152.6%	108.8%*	93.0%*
	1 µL Rec.	-	-	33.9%	9.8%	94.4%	-	-

5 µL level concentrations in pg/mL. BBA = bead-based assay. Rec. = recovery. *Indicates lowest and greatest volume at which linearity of dilution recovery was within 30% of the 5 µL value. Buffer: Teknova assay buffer with anti-protease (TAB-AP).

Tables 34 and 35 show spike-recovery for NS (Subject 23) and STIM (Subjects 15 and 23) tear samples. For NS and STIM tears on both plates, the best recovery was at low spike levels. High and medium spikes produced much better recovery on the polystyrene plate than on the magnetic plate for both tear types. Overall, NS showed better spike-recovery than STIM tears and the polystyrene plate produced better spike-recovery than the magnetic plate. This indicates that NS tears produce the most reliable results when coupled with polystyrene BBA.

Table 34a

Spike-recovery for Non-stimulated Tear Samples on Polystyrene and Magnetic BBAs

		IL-1β	IL-1ra	IL-2	IL-4	IL-5	IL-6	IL-7	IL-8	IL-9	IL-10	IL-12p70
	Recovery					Polystyrene Bead Plate						
Subject 23	High Spike	34.2%	84.2%	8.5%	19.2%	51.4%	22.0%	50.1%	65.6%	76.8%	55.4%	28.8%
	Medium Spike	41.4%	85.8%	24.5%	48.1%	56.4%	52.9%	58.6%	82.1%	72.5%	76.3%	34.3%
	Low Spike	37.0%	89.8%	79.8%	93.8%	78.9%	80.4%	75.4%	96.6%	83.3%	91.7%	58.3%
						Magnetic Bead Plate						
Subject 23	High Spike	4.3%	19.1%	8.3%	12.6%	7.2%	8.0%	5.0%	28.2%	12.4%	10.1%	8.2%
	Medium Spike	8.2%	71.9%	22.2%	39.7%	15.9%	17.5%	12.6%	65.7%	49.3%	17.5%	19.1%
	Low Spike	31.7%	111.7%	72.1%	89.8%	51.9%	71.4%	61.2%	99.0%	100.5%	42.8%	67.6%

BBA = bead-based assay. Buffer: Teknova assay buffer with anti-protease (TAB-AP).

Table 34b
Spike-recovery for Non-stimulated Tear Samples on Polystyrene and Magnetic BBAs

		IL-13	IL-15	IL-17	Eotaxin	FGF-basic	G-CSF	GM-CSF	IFN-γ	IP-10	MCP-1	MIP-1α
	Recovery					Polystyrene Bead Plate						
Subject 23	High Spike	48.7%	24.9%	88.7%	51.0%	-	35.2%	44.0%	26.3%	64.1%	63.3%	48.1%
	Medium Spike	65.6%	46.0%	78.7%	60.1%	-	36.7%	54.0%	60.4%	107.2%	67.8%	63.1%
	Low Spike	65.9%	51.9%	77.5%	80.8%	-	57.2%	95.4%	101.9%	84.2%	77.6%	82.6%
						Magnetic Bead Plate						
Subject 23	High Spike	11.5%	3.3%	6.7%	22.3%	47.1%	6.5%	11.0%	17.6%	25.9%	7.7%	10.0%
	Medium Spike	15.8%	7.4%	17.8%	36.1%	68.8%	11.5%	43.9%	65.0%	74.5%	25.1%	28.3%
	Low Spike	43.8%	23.7%	69.6%	82.4%	126.4%	55.7%	93.7%	105.2%	98.3%	84.3%	78.5%

BBA = bead-based assay. Buffer: Teknova assay buffer with anti-protease (TAB-AP).

Table 34c

Spike-recovery for Non-stimulated Tear Samples on Polystyrene and Magnetic BBAs

		MIP-1β	PDGF-bb	RANTES	TNF-α	VEGF
	Recovery			Polystyrene Bead Plate		
Subject 23	High Spike	-	45.0%	17.8%	67.2%	35.4%
	Medium Spike	-	44.6%	43.8%	71.2%	65.7%
	Low Spike	-	50.8%	92.9%	95.2%	94.1%
				Magnetic Bead Plate		
Subject 23	High Spike	11.8%	13.7%	12.9%	16.5%	6.8%
	Medium Spike	31.0%	15.8%	40.9%	62.4%	23.8%
	Low Spike	83.0%	44.5%	94.3%	112.0%	81.6%

BBA = bead-based assay. Buffer: Teknova assay buffer with anti-protease (TAB-AP).

Table 35a

Spike-recovery for Stimulated Tear Samples on Polystyrene and Magnetic BBAs

		IL-1β	IL-1ra	IL-2	IL-4	IL-5	IL-6	IL-7	IL-8	IL-9	IL-10	IL-12p70
	Recovery						Polystyrene Bead Plate					
Subject 23	High Spike	34.6%	60.5%	4.6%	13.4%	37.8%	31.9%	35.1%	36.9%	55.9%	46.7%	31.8%
	Medium Spike	51.6%	83.9%	31.4%	53.2%	43.3%	49.4%	48.0%	61.2%	76.6%	68.5%	47.0%
	Low Spike	66.8%	100.7%	82.5%	100.0%	80.1%	86.5%	78.6%	91.4%	85.7%	101.7%	80.8%
							Magnetic Bead Plate					
Subject 23	High Spike	2.3%	20.2%	6.5%	11.0%	3.8%	5.5%	3.0%	21.3%	14.2%	5.5%	6.1%
	Medium Spike	10.4%	86.8%	29.6%	57.8%	19.7%	27.1%	19.2%	77.5%	73.0%	17.7%	24.8%
	Low Spike	51.3%	114.8%	89.6%	100.6%	70.4%	89.8%	79.3%	111.3%	109.6%	65.9%	82.9%
Subject 15	High Spike	2.5%	13.9%	6.2%	10.1%	3.9%	4.8%	3.0%	19.4%	15.2%	5.5%	5.8%
	Medium Spike	7.4%	63.5%	21.1%	41.7%	13.5%	18.3%	13.2%	55.0%	58.2%	14.1%	18.5%
	Low Spike	30.5%	88.4%	67.3%	78.2%	49.4%	64.3%	53.1%	83.6%	85.1%	41.8%	60.4%

BBA = bead-based assay. Buffer: Teknova assay buffer with anti-protease (TAB-AP).

Table 35b

Spike-recovery for Stimulated Tear Samples on Polystyrene and Magnetic BBAs

		IL-13	IL-15	IL-17	Eotaxin	FGF-basic	G-CSF	GM-CSF	IFN-γ	IP-10	MCP-1	MIP-1α
	Recovery					Polystyrene Bead Plate						
Subject 23	High Spike	42.1%	31.4%	75.3%	34.2%	121.5%	30.1%	34.8%	18.8%	52.0%	73.1%	31.7%
	Medium Spike	67.0%	46.1%	74.2%	61.8%	103.9%	40.2%	78.9%	89.4%	75.2%	81.4%	60.7%
	Low Spike	85.1%	59.2%	94.8%	89.9%	73.6%	74.3%	98.5%	109.9%	95.0%	89.2%	86.9%
						Magnetic Bead Plate						
Subject 23	High Spike	6.7%	2.3%	4.9%	16.8%	39.7%	4.1%	12.6%	19.6%	12.2%	6.8%	6.3%
	Medium Spike	15.7%	9.4%	29.1%	44.7%	84.3%	18.6%	62.2%	89.1%	61.2%	39.4%	31.6%
	Low Spike	55.1%	39.5%	83.2%	90.5%	109.1%	76.9%	110.5%	110.5%	101.8%	93.5%	85.3%
Subject 15	High Spike	6.3%	2.3%	4.4%	17.5%	39.6%	3.8%	10.0%	15.6%	15.7%	6.0%	6.8%
	Medium Spike	13.7%	6.6%	18.9%	35.0%	69.8%	11.8%	48.3%	65.9%	58.4%	27.3%	27.1%
	Low Spike	38.9%	22.2%	65.4%	72.7%	92.7%	46.7%	81.8%	90.0%	85.0%	68.5%	71.3%

BBA = bead-based assay. Buffer: Teknova assay buffer with anti-protease (TAB-AP).

Table 35c

Spike-recovery for Stimulated Tear Samples on Polystyrene and Magnetic BBAs

		MIP-1β	PDGF-bb	RANTES	TNF-α	VEGF
	Recovery			Polystyrene Bead Plate		
Subject 23	High Spike	-	76.0%	12.8%	59.5%	25.4%
	Medium Spike	-	86.2%	48.9%	89.3%	58.0%
	Low Spike	-	98.6%	94.3%	94.4%	100.2%
				Magnetic Bead Plate		
Subject 23	High Spike	7.4%	8.2%	11.9%	18.2%	4.3%
	Medium Spike	28.9%	32.5%	53.6%	78.8%	29.6%
	Low Spike	83.0%	88.6%	99.2%	101.6%	87.2%
Subject 15	High Spike	7.3%	8.2%	10.6%	14.3%	4.4%
	Medium Spike	23.8%	21.2%	43.3%	62.2%	20.1%
	Low Spike	69.0%	62.7%	85.8%	87.6%	62.3%

BBA = bead-based assay. Buffer: Teknova assay buffer with anti-protease (TAB-AP).

27-Plex Polystyrene and Magnetic BBA (Flowchart #12)

The optimal buffer, TAB-AP, and optimal conditions, filter plate and automatic wash/aspiration system, were used to do a final comparison of the polystyrene BBA to the magnetic BBA. NS, WO, and STIM tear samples were run using Bio-Rad 27-Plex polystyrene and magnetic BBA kits. A total of 20 subjects (normal: 1, 2, 4, 5, 6, 7, 8, 9, 10, 11, 12; dry: 15, 16, 17, 19, 20, 21, 22, 23, 24) collected a WO sample immediately followed by a STIM sample in order to compare these tear sample types on a polystyrene and magnetic plate (Table 36). Five of these 20 subjects (9, 11, 19, 21, and 24) plus one additional subject (13) collected a NS and WO sequence for a similar comparison (Table 37). WO tears diluted in TAB gave similar results to NS tears diluted in TAB. Because WO tears could be collected at larger volumes than NS tears in a much shorter period of time and such a large volume of tears were required for these plates, more WO tear samples were used. Samples were pooled before each assay to be able to compare results accurately.

Cytokine concentrations were higher for WO samples on the polystyrene plate than the magnetic plate for 23 out of 27 cytokines and greater for STIM samples on the polystyrene plate for 21 out of 27 cytokines (Table 36). Similar results were also seen in the NS and WO comparisons (Table 37). For NS, 20 cytokines were higher with polystyrene than magnetic and for WO, 19 cytokines were higher with polystyrene than magnetic. In addition, of the six subjects whose NS and WO samples were sequentially collected, FGF-basic was not detected. When compared to published cytokine values, 18

polystyrene BBA cytokines showed concentrations closer to those reported by LaFrance et al [46] compared to the magnetic BBA.

The polystyrene plate had a lower LLOQ than the magnetic plate for 18 cytokines and a greater ULOQ than the magnetic plate for 11 cytokines (Table 38). The LLOQ is more important than the ULOQ because most cytokine concentrations of tear samples are typically quantified closer to the lower limit. Only a few cytokines, such as IP-10, are detected in the higher range near ULOQ. As has been seen in previous assays in this study, polystyrene appears to be a better BBA than magnetic because it is more sensitive than magnetic at the lower levels of quantification.

Comparison of polystyrene and magnetic BBAs: Overall cytokine profile. It was important to determine if the two assays were measuring the same thing. A single subject's levels of 27 different cytokines were compared using polystyrene and magnetic BBAs (Figure 9). There is expected variation of cytokine levels within this subject's tear samples using both assay types. On average, the magnetic BBA measured 63% of the polystyrene BBA for NS tears (Figure 9a) and 53% of the polystyrene BBA for STIM tears (Figure 9b). However, based on the correlations of coefficients, the overall cytokine profiles were similar for both assay types with NS and STIM tears.

Table 36

27-Plex Polystyrene and Magnetic BBAs: WO and STIM

	Polystyrene		Magnetic	
Cytokine	WO	STIM	WO	STIM
IL-1β	1.77	2.43	2.52	3.85
IL-1ra	3,130.25	969.44	401.20	373.48
IL-2	19.12	28.43	2.13	3.62
IL-4	8.66	12.51	7.32	9.54
IL-5	3.16	6.14	7.34	9.72
IL-6	19.52	19.58	12.51	15.91
IL-7	87.01	94.54	27.32	30.25
IL-8	141.79	130.48	58.09	55.91
IL-9	943.58	96.79	692.19	58.27
IL-10	95.77	102.29	5.59	6.70
IL-12p70	41.00	44.11	57.57	60.45
IL-13	10.53	13.16	12.31	13.05
IL-15	2.84	2.44	0.26	0.64
IL-17	13.88	11.40	4.98	7.19
Eotaxin	80.61	94.05	22.11	31.28
FGF basic	30.32	24.73	3.64	5.32
G-CSF	26.60	12.02	21.58	30.34
GM-CSF	43.23	83.26	13.77	-
IFN-γ	108.32	181.47	68.19	102.41
IP-10	21,515.75	14,832.83	1,696.74	1,359.25
MCP-1	36.79	17.91	5.09	8.58
MIP-1α	47.29	60.33	18.92	23.15
MIP-1β	83.21	-	9.74	10.52
PDGF-bb	11.49	14.00	8.57	14.43
RANTES	80.24	99.95	56.18	74.92
TNF-α	246.86	381.01	162.50	259.70
VEGF	326.55	316.15	31.22	28.20

Tear cytokine concentrations in pg/mL. BBA = bead-based assay. WO = washout tears. STIM = stimulated tears. 20 subjects. Buffer: Teknova assay buffer with anti-protease (TAB-AP).

Table 37

27-Plex Polystyrene and Magnetic BBAs: NS and WO

	Polystyrene		Magnetic		27-Plex PS BBA (NS)
Cytokine	NS	WO	NS	WO	LaFrance [46]
IL-1β	2.8	2.6	2.8	2.9	5.2
IL-1ra	4,277.7	2,963.1	492.3	528.7	9,589.4
IL-2	17.4	21.3	-	1.1	61.9
IL-4	7.3	8.0	7.1	7.6	29.3
IL-5	1.9	2.8	7.3	8.1	13.7
IL-6	36.5	21.1	15.1	13.6	35.2
IL-7	99.2	95.1	27.6	26.5	382.3
IL-8	1,014.5	620.8	231.3	194.4	147.7
IL-9	28.9	25.1	12.0	16.7	35.6
IL-10	109.6	105.0	5.5	6.1	23.6
IL-12p70	39.7	39.0	57.7	57.3	32.9
IL-13	10.9	11.0	12.0	11.9	19.6
IL-15	3.2	1.8	0.01	0.2	14.0
IL-17	-	1.6	2.5	4.5	72.2
Eotaxin	59.7	45.3	8.6	17.2	293.7
FGF basic	-	-	-	-	-
G-CSF	27.4	13.6	26.1	22.5	43.3
GM-CSF	23.4	33.5	-	-	95.7
IFN-γ	93.7	124.1	68.3	73.2	331.6
IP-10	15,321.6	18,942.1	2,275.8	2,262.4	23,622.3
MCP-1	54.2	57.1	4.6	4.7	132.5
MIP-1α	32.5	35.1	12.3	16.8	26.2
MIP-1β	-	-	10.6	8.8	48.5
PDGF-bb	15.0	14.5	7.8	8.5	31.6
RANTES	81.3	79.8	57.3	55.5	36.1
TNF-α	198.5	194.7	122.4	189.1	175.9
VEGF	467.8	411.8	42.9	45.4	2,608.5

Tear cytokine concentrations in pg/mL. BBA = bead-based assay. NS = non-stimulated tears. WO = washout tears. PS = polystyrene. 6 subjects. Buffer: Teknova assay buffer with anti-protease (TAB-AP).

Table 38

Limits of Quantification: Final Comparison of Polystyrene and Magnetic

	Polystyrene		Magnetic	
Cytokine	LLOQ	ULOQ	LLOQ	ULOQ
IL-1β	0.63	840.94	0.64	1,044.47
IL-1ra	0.89	13,926.74	0.98	14,632.98
IL-2	0.33	1,483.40	0.34	4,979.36
IL-4	0.06	1,115.09	0.07	1,054.74
IL-5	0.59	224.38	0.58	881.38
IL-6	0.50	8,322.21	0.50	7,218.72
IL-7	0.55	694.50	0.57	6,132.08
IL-8	0.45	9,272.67	0.54	629.71
IL-9	2.20	8,768.68	7.21	8,848.62
IL-10	0.52	7,119.61	0.50	663.12
IL-12p70	0.58	10,348.68	0.74	9,791.50
IL-13	0.73	1,085.40	0.73	250.00
IL-15	0.58	689.98	0.57	968.65
IL-17	0.42	7,142.22	0.48	5,691.07
Eotaxin	6.78	7,906.75	5.88	6,578.76
FGF basic	2.91	3,989.99	0.68	3,492.12
G-CSF	0.55	2,770.25	0.59	6,519.65
GM-CSF	0.76	3,966.17	3.53	4,129.80
IFN-γ	0.39	6,851.41	1.21	7,108.00
IP-10	0.73	928.58	0.87	8,795.76
MCP-1	0.41	4,987.01	0.46	5,931.02
MIP-1α	1.26	436.36	1.08	1,429.60
MIP-1β	2.80	371.64	0.39	458.95
PDGF-bb	0.43	5,603.42	0.46	6,294.34
RANTES	0.39	542.77	1.66	4,479.50
TNF-α	4.35	22,938.40	21.11	22,696.00
VEGF	0.48	9,753.58	0.68	9,051.87

Tear cytokine concentrations in pg/mL. BBA = bead-based assay. LLOQ = lower limit of quantification. ULOQ = upper limit of quantification. Buffer: Teknova assay buffer with anti-protease (TAB-AP).

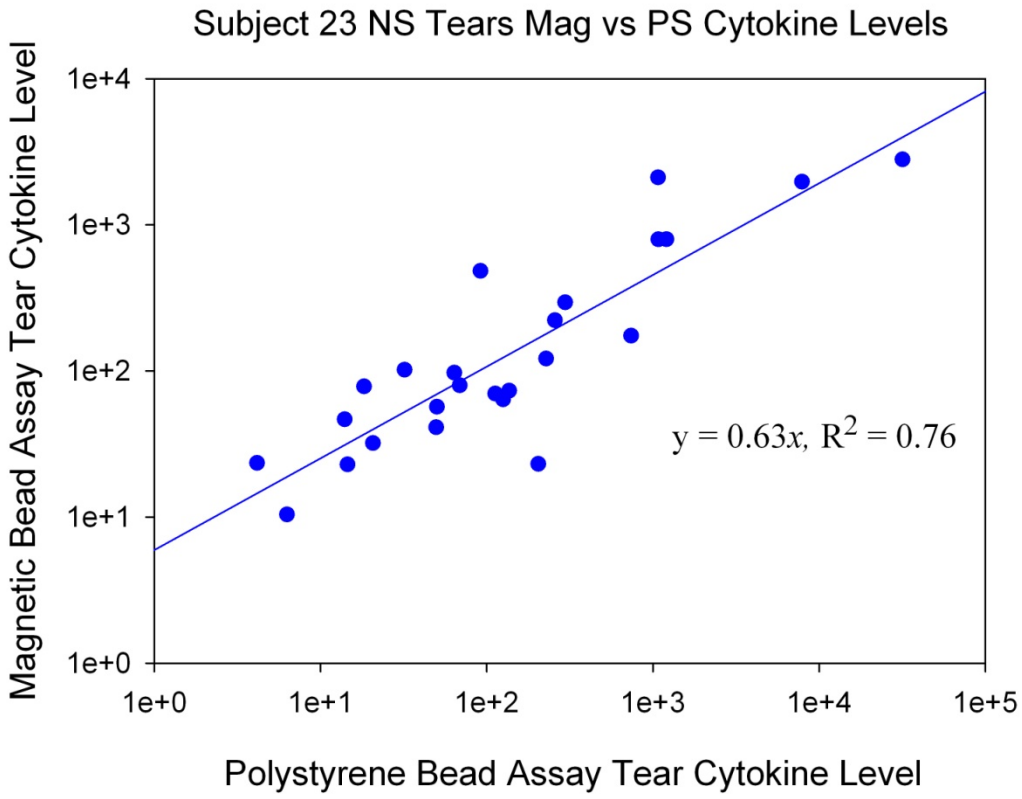

Figure 9a. Overall Cytokine Profile within a Single Subject (Subject 23): Magnetic versus Polystyrene Bead-based Assay (Non-stimulated Tears)

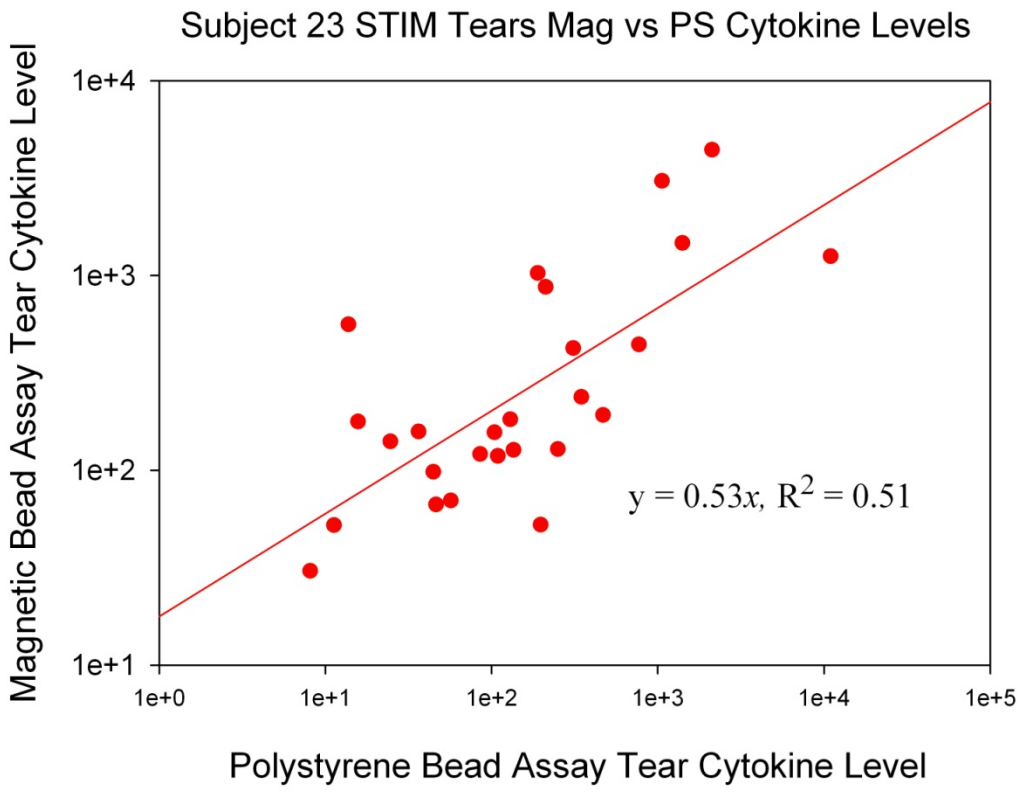

Figure 9b. Overall Cytokine Profile within a Single Subject (Subject 23): Magnetic versus Polystyrene Bead-based assay (Stimulated Tears)

Comparison of polystyrene and magnetic BBAs: Individual cytokine levels. The individual cytokine levels of IL-6 (Figure 10) and IL-8 (Figure 11) were measured across multiple subjects included in the final assay comparisons. Each figure shows comparisons of the two assay types using NS, WO, or STIM tear samples. In all figures, when the polystyrene BBA detection was near zero, the magnetic BBA produced a positive cytokine reading. A matrix effect within one of the assays is likely causing this difference. Of the

three tear sample types, NS tears had the greatest positive reading (Figures 10a and 11a) suggesting it had the strongest matrix effect.

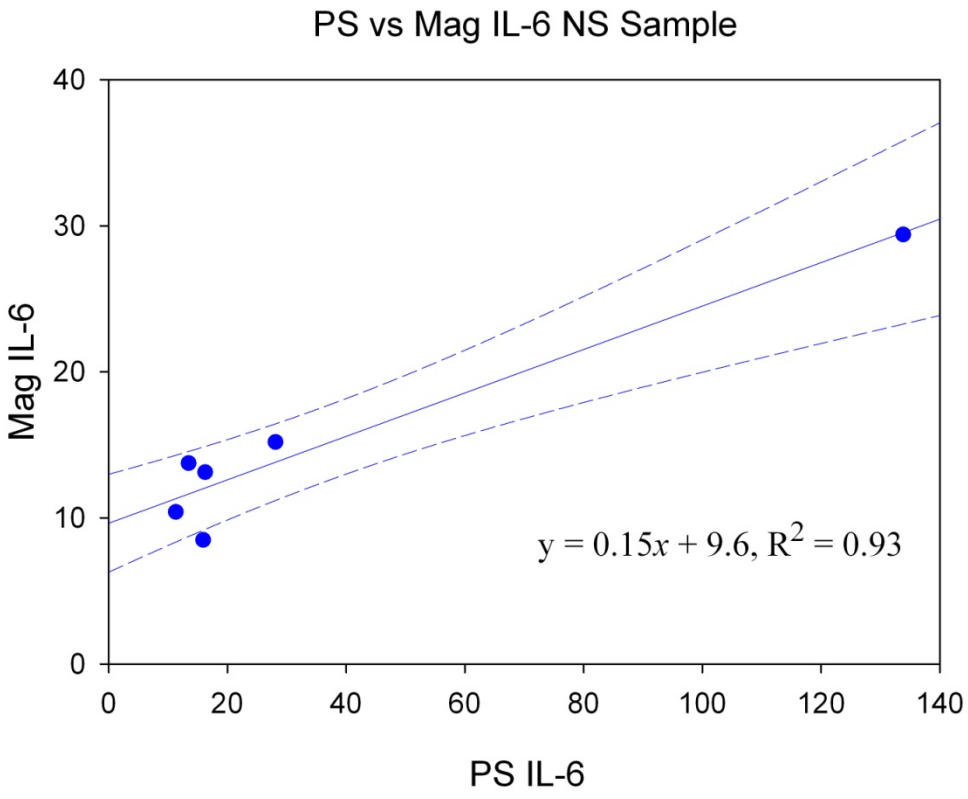

Figure 10a. Level of One Cytokine (IL-6) for 6 Subjects: Magnetic versus Polystyrene Bead-based Assay (Non-stimulated Tears)

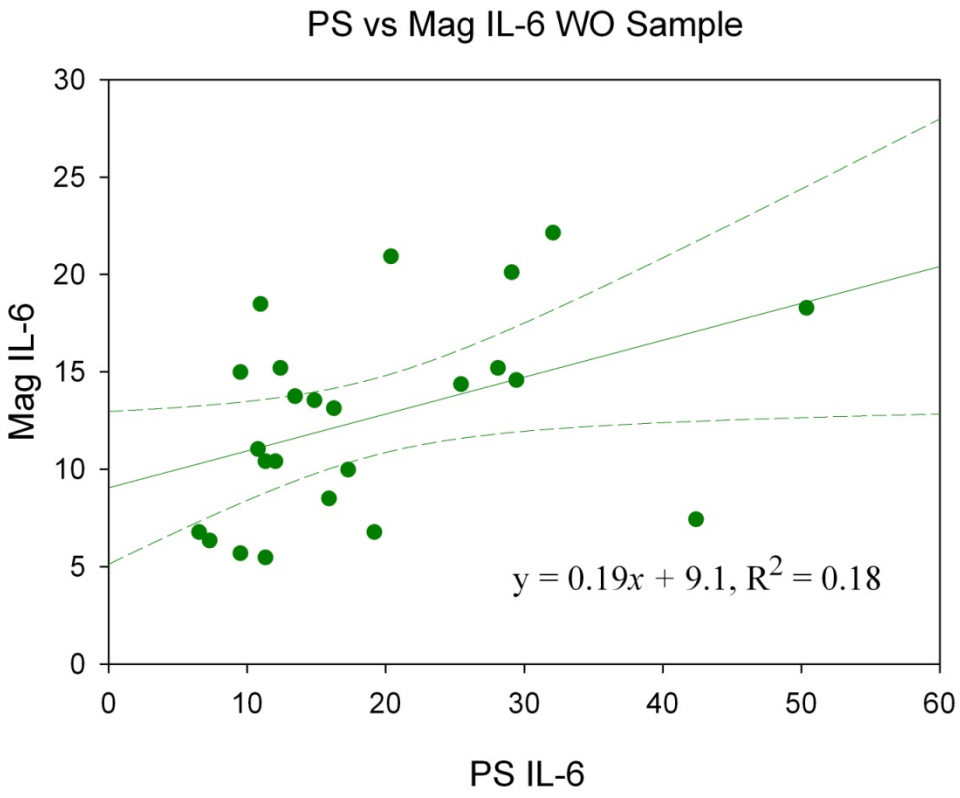

Figure 10b. Level of One Cytokine (IL-6) for 24 Subjects: Magnetic versus Polystyrene Bead-based Assay (Washout Tears)

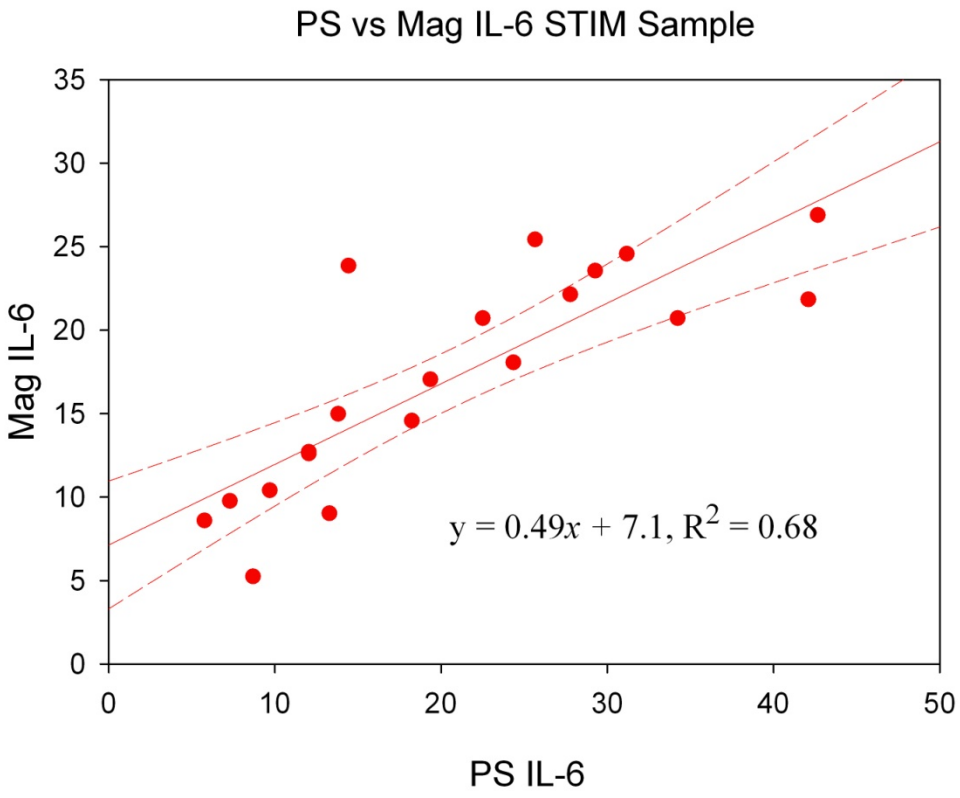

Figure 10c. Level of One Cytokine (IL-6) for 19 Subjects: Magnetic versus Polystyrene Bead-based Assay (Stimulated Tears)

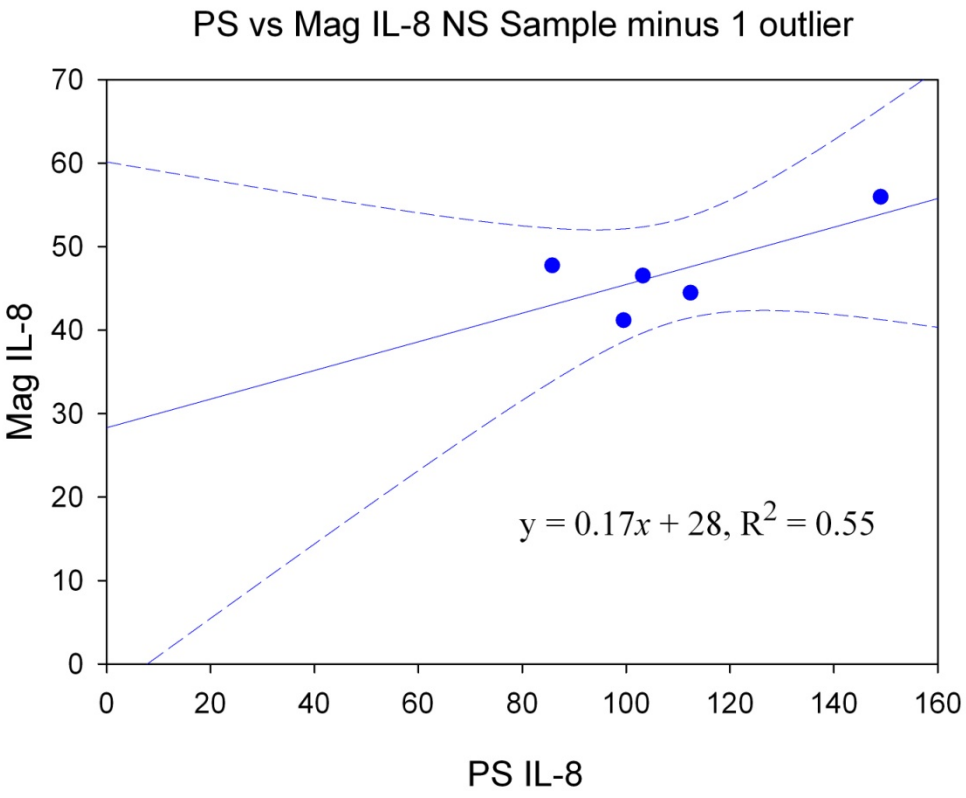

Figure 11a. Level of One Cytokine (IL-8) for 5 Subjects: Magnetic versus Polystyrene Bead-based Assay (Non-stimulated Tears)

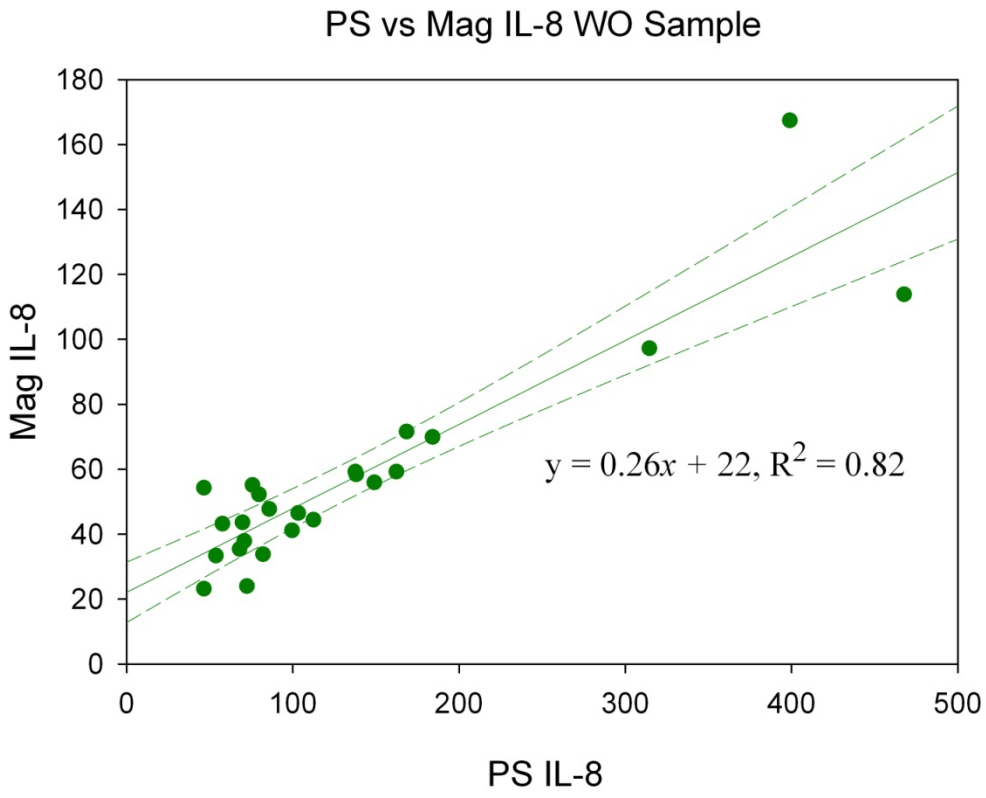

Figure 11b. Level of One Cytokine (IL-8) for 23 Subjects: Magnetic versus Polystyrene Bead-based Assay (Washout Tears)

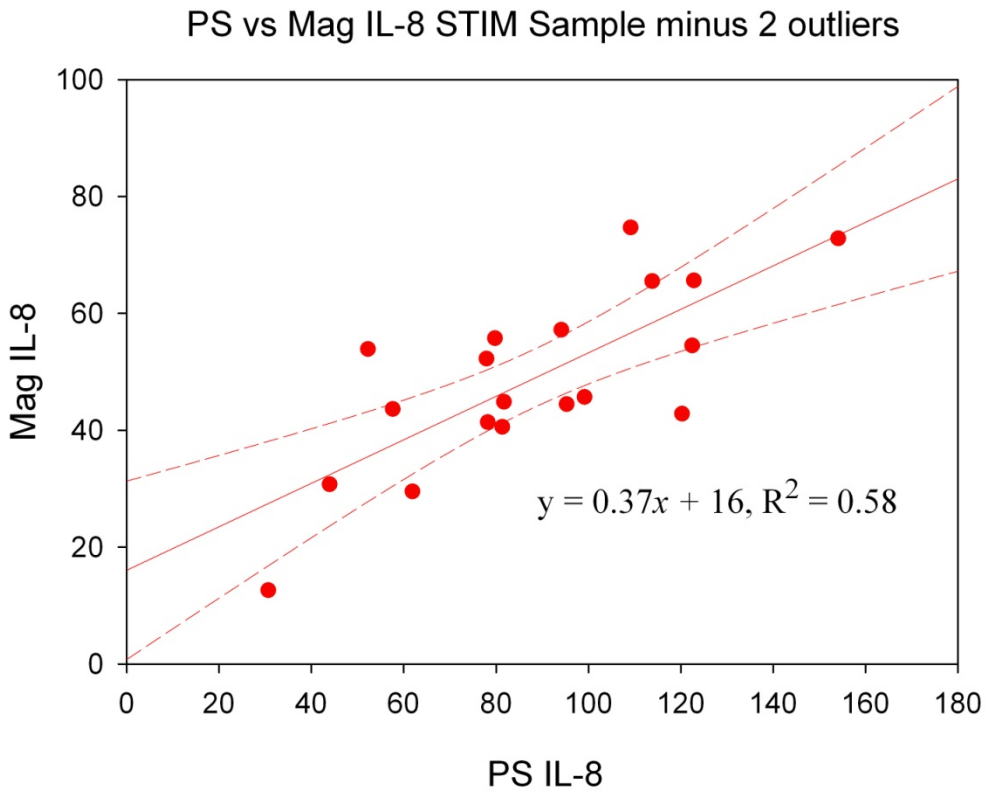

Figure 11c. Level of One Cytokine (IL-8) for 19 Subjects: Magnetic versus Polystyrene Bead-based Assay (Stimulated Tears)

DISCUSSION

The series of experiments conducted in this study have resulted in a substantially improved magnetic BBA. However, the polystyrene bead version remains the better assay, having greater sensitivity and a less significant matrix effect.

To begin this study, the Luminex instrument, which is the system used to evaluate tear samples, assessed the ability of BBAs to give acceptable results. Results from any data collected in this study would be invalid if the system was not functioning properly. For this evaluation, we compared tear samples diluted in two different buffers (MAB and MSB) on a 13-Plex polystyrene LINCOplex BBA. The outcome of the buffer comparison was that MAB produced superior results because it detected more cytokines within the assay working range. The range of detection of cytokines for most subjects' tear samples was comparable to those found in previous studies by LaFrance [46]. Therefore, the conclusion was that the Luminex system could run further BBAs.

Because the Luminex instrument was giving acceptable results, the available filter plate washing/aspiration system were next evaluated next using a Bio-Rad 27-Plex polystyrene BBA kit. The optimal washing system will help limit intra- and inter-assay variation. The manual and automatic wash systems each have positive and negative features. First, the manual system requires the use of many racks of pipette tips for application of wash buffer. In addition, variation can occur if application of wash buffer to wells is un-

even or if the vacuum pressure varies across the plate. Positive characteristics of the manual system include less residual volume after vacuuming of the filter plate. On the other hand, with the automatic plate washer, residual volumes accumulate beneath the plate between vacuum steps, which may decrease assay efficiency and encourage wicking. However, the automatic plate washer does not require any pipette tips. Uniform vacuum pressure and wash buffer dispensing across the plates should improve intra- and inter-assay variation. This system eliminates the issues of the applying of varying volumes of wash buffer and non-uniform vacuum pressure.

BBA results with the manual and automatic plate washer were similar in terms of cytokine detection and CVs. However, the limits of quantification were narrower than usual for these BBAs because of the decision to use only four standards. Therefore, some cytokines that would normally be within the limits of quantification were outside of this range. The remaining BBA studies used the automatic system because neither system appeared to be better than the other was. Automation has the advantage of removing one potential source of human error.

After determining the automatic wash/aspiration system was best for the tear BBAs, comparison of 3 different buffers (TAB, MAB, and BAB) using a Bio-Rad 27-Plex polystyrene BBA worked towards the goal of determining the optimal buffer. Inclusion of BAB occurred because it is the buffer recommended by Bio-Rad in the assay kit for sample dilution. In the past, the primary buffer for sample dilution in this lab was MAB. TAB was included in the current comparison because its composition may be similar to MAB and it more readily available.

The results of this three-buffer comparison showed very different results for BAB compared to both TAB and MAB. BAB produced greater tear cytokine concentrations along with reduced assay sensitivity. The considerably larger concentrations with BAB may have been due to its lack of a serum component to act as a blocking agent. TAB and MAB both contain a serum component. Serum composition appears to be important and may have been the reason for concentration differences. Serum-containing buffers have been shown to block non-specific binding in other studies [45].

TAB and MAB also showed interesting results. These two buffers should behave similarly because each contains BSA. The results, however, showed a definite lack of equivalence because a number of cytokines had much lower limits of detection with TAB than MAB (Table 11). This indicates that TAB has greater potential to quantify cytokines in smaller (sub-5μL) tear volumes than MAB. However, the differences were not great enough to choose TAB as the single optimal buffer at this stage. Further buffer studies were necessary.

In the next study, comparison of tear samples diluted in TAB and BBS, a custom-made buffer containing multiple blocking agents (Table 6), occurred using a Bio-Rad 27-Plex polystyrene BBA. BBS contains normal mouse serum in addition to BSA. Non-immune mouse serum has been shown to be effective at blocking non-specific binding in assays, so normal mouse serum was included in BBS to see if it would have a similar effect [45]. In general, TAB appeared to be more sensitive than BBS. However, BBS produced lower levels of detection than TAB in some cases. Linearity of dilution was also determined for the two subjects involved in this assay (Table 13). This is an important

measure because it indicates the lowest volume of tears that can provide an accurate reading. In Subject 1, each buffer produced similar linearity recovery levels, but for different cytokines. Although there were similarities in recovery levels, TAB yielded detection at lower tear volumes than BBS for many cytokines. This means that BBS is not as sensitive as TAB in this assay. Because TAB again yielded superior results in the polystyrene BBA, TAB became the key buffer for the next stage, investigation of magnetic BBA performance.

The first Bio-Rad magnetic BBA, using TAB as assay buffer, compared filter and plastic assay plates. A single-plex IL-8 assay was chosen because it has a history of interference in ELISA and polystyrene BBAs demonstrated in this lab [35]. The choice to assay only one cytokine also circumvented any interference between bead populations. Magnetic beads require either a filter or a plastic plate. Comparison of the two magnetic bead plate types contributed to reaching one of the study objectives of obtaining magnetic BBA results that are equivalent to polystyrene BBAs. The plastic plate should be the better plate type because there would be no possible decrease in efficiency from residual volume after vacuuming. In addition, it would not require manual removal of residual liquid because no vacuum steps are involved.

Results from this magnetic BBA were generally better with the filter plate. Bead aggregation was much greater for the plastic plate. The differences could be due to magnetic beads binding non-specifically to the plastic plate surface. Both plates showed lower bead counts than past polystyrene BBAs run in this lab. This was fully expected because the magnetic BBA uses lower bead volumes per well. The plastic plate showed

higher IL-8 levels than the filter plate. Tear samples on the filter plate elicited better linearity of dilution than the plastic plate. The fact that the filter plate assay had better linearity of dilution and therefore greater potential to work with smaller tear volumes was the key to choosing it over the plastic plate.

Another Bio-Rad single-plex (IP-10) magnetic BBA continued the process of exploring magnetic BBAs. Similar to IL-8, IP-10 has shown interference in past ELISA and polystyrene BBA studies in this lab [35]. The buffers chosen for this assay were TAB and BBS because TAB had given results comparable to previous assays and in order to test BBS on a magnetic bead plate and. Both buffers showed similar linearity of dilution, indicating that each buffer was capable of detecting similar levels of cytokines in lower volume tear samples. The main difference in this assay was that IP-10 levels in TAB were comparable to earlier studies in this lab but BBS concentrations were not comparable and were much greater than TAB. This could have been due to non-specific binding between the beads and tear samples or matrix effects causing the elevated cytokine levels with BBS. Again, BBS appears to be a less optimal buffer because IP-10 concentrations were much greater than accepted values using this 1-Plex magnetic BBA. Results with TAB were comparable to earlier ELISA, the gold standard.

After running two Bio-Rad 1-Plex magnetic BBAs (IL-8 and IP-10), a 3-Plex magnetic BBA composed of IL-8, IFN-γ, and IP-10 was run in order to gradually increase the plex, or number of cytokines assayed, and continue buffer comparisons. IFN-γ was included in the 3-Plex BBA because it functions as an inducer of IP-10. There were three buffers used for this assay: TAB, DAB, and BSD. DAB was chosen for comparison

because it was used in the ELISA studies that have been compared throughout this paper [35]. BSD was included because it has a serum component that, as mentioned earlier, may decrease non-specific binding.

Results from this 3-Plex magnetic BBA were not as conclusive as other assays run up to this point. IFN-γ levels with TAB were below and levels with DAB and BSD were above previous polystyrene BBA data. Variability was lowest for IP-10 with TAB when compared to the DAB and BSD. In addition, IP-10 levels with TAB were closer to those found with previous ELISA and polystyrene BBAs than the other buffers. DAB showed the best linearity of dilution amongst the buffers. None of the buffers used in this 3-Plex magnetic BBA produced data that was consistent enough with previous data to consider it superior to the others. Therefore, further testing of buffers was necessary. Further expansion to larger plex magnetic BBAs was the logical step for this exploration of buffers to find the one whose cytokine levels were most consistent with published polystyrene BBA data.

A Bio-Rad 11-Plex magnetic BBA was next run. This assay contained matched NS and STIM tear samples from four subjects diluted in TAB and DAB. Again, more cytokines are included with magnetic kits in order to note any differences as the number of assayed cytokines increases and to compare to previous polystyrene BBAs. TAB and DAB were the buffers used for this assay because the results of magnetic BBAs run so far had not shown TAB as a definite choice for the optimal buffer and DAB had shown some positive results in the 3-Plex magnetic BBA. BSD was not included on this plate because it showed the least favorable results on the 3-Plex magnetic BBA.

In most cases for the 11-Plex BBA, TAB showed better linearity of dilution than DAB. This means smaller volume tear samples stored in TAB will enable more cytokine quantification than those stored in DAB. A paired t-test of NS and STIM tear samples showed significantly greater cytokine concentrations with DAB than TAB. There were very similar ranking of cytokines from NS and STIM tear samples stored in each buffer indicating that the two were equally capable of providing cytokine profiles. This result does not make one better appear better than the other. Concentrations for most samples stored in TAB in this assay were closer to those found in previously published polystyrene BBA data.

Because TAB appeared to be the superior buffer in three of the five assays that compared it to other buffers [(27-Plex polystyrene BBA: TAB vs. BBS (#4); IP-10 magnetic BBA: TAB vs. BBS (#6); 11-Plex magnetic BBA: TAB vs. DAB (#8)], TAB was the optimal buffer choice for tear BBAs. Anti-protease cocktail tablets when added to TAB can optimize the blocking effect by inhibiting breakdown of cytokines after tear collection.

Tear samples diluted in TAB-AP were run on a Bio-Rad 27-Plex polystyrene BBA and showed slightly lower concentrations than samples diluted in TAB without AP. This reduction in cytokine levels may have occurred due to anti-proteases in TAB-AP inhibiting non-specific binding to beads. The difference was not great enough to consider the addition of anti-protease to be detrimental to assay performance. Anti-protease was therefore included in all subsequent assays.

The next step towards obtaining similar results for polystyrene and magnetic BBAs involved different tear sample types, NS, WO, and STIM. These showed how TAB would affect the quantification of each. Using a Bio-Rad 27-Plex magnetic BBA kit, TAB quantified the tear samples at greater concentrations in the magnetic BBA than previously published polystyrene data [38]. Because statistical analysis showed a significant difference between the assay types for NS tears, it was the first indication that the magnetic BBA may not be able to provide equivalent results to the polystyrene BBA for tear assays. This finding is important because the Bio-Rad polystyrene BBA kit, which has given acceptable results for tear assays in this lab, is no longer available. Only magnetic BBA kits are now available.

Linearity of dilution and spike-recovery for the pooled STIM samples of one normal subject allowed further comparison of Bio-Rad 27-Plex polystyrene and magnetic BBAs. As stated earlier, linearity of dilution is an important measure of sensitivity because it determines the lowest tear volume for tear cytokine quantification. Spike-recovery is important because it determines how well an assay can detect a known concentration of standard in a tear sample. If recovery is less or greater than 100%, a matrix effect due to a buffer-tear component interaction is implicated. The magnetic particles on the bead surface may also contribute to the matrix effect.

Linearity of dilution was much better for the polystyrene plate when compared to the magnetic plate. Not only did the polystyrene plate produce better linearity, it also detected more cytokines than the magnetic plate. Linearity could not be determined for some cytokines in the magnetic BBA because there was no detection at the 5µL level.

Spike-recovery produced questionable results with much lower percent recovery for high and medium spikes on the magnetic plate than the polystyrene. A very likely reason for lower spike-recovery was the subject is intrinsically low for many cytokines. By interpolating from the low end of the standard curve range, recoveries would be far from optimal. This result led to the decision to run additional BBAs of tear samples from subjects with higher intrinsic cytokine levels. These BBAs again showed lower recovery for high and medium spikes on the magnetic plate when compared to the polystyrene plate. Spike-recovery was better for NS than STIM tears. This is expected because NS tears generally have higher intrinsic cytokine levels [46]. Samples with larger tear volumes were also included in these BBAs to see if viable results were possible at tear levels exceeding 5µL. Results did not show linear increases in concentrations as the tear volume increased beyond 5µL. Therefore, large tear volumes would not produce valid results on either assay type presumably due to increased matrix effects. Overall, the polystyrene BBA showed better linearity of dilution and spike-recovery than the magnetic BBA. The polystyrene BBA continued to show more promising results than the magnetic BBA in almost all comparisons.

A final comparison of Bio-Rad 27-Plex polystyrene and magnetic BBA kits involved the optimal buffer, TAB-AP, and optimal conditions, the automatic wash/aspiration system and filter plate. NS, WO, and STIM tear samples all showed lower cytokine concentrations with the magnetic plate than the polystyrene plate. This trend toward lower cytokine values with the magnetic plate was a common theme throughout the latter part of this study. Based on spike-recovery results, matrix effects appear to in-

terfering with or blocking the binding of tear samples to antibody-bound beads causing a reduced cytokine level with the magnetic BBA.

Another way that the polystyrene BBA appears to be better than the magnetic BBA involves limit of quantification. This is an important measure because it determines the upper and lower limits within which accurate quantification of tears can occur. In these final two BBAs, the polystyrene BBA had more cytokines with a lower LLOQ than the magnetic BBA and had similar ULOQ results for each. This is significant because most detection of tear cytokines occurs at levels very close to the lower limit. Quantification of IP-10 is often closer to the upper limit but very few other cytokines ever approach the upper limit.

Comparing the final polystyrene and magnetic BBAs, NS cytokine concentrations with the magnetic BBA differed from previously published polystyrene BBA results [46]. This finding is very important to the overall study because it shows that the magnetic BBA is continuing to yield results that are not equivalent to the validated polystyrene BBA data.

Important to the cumulative analysis of the study is whether the two assay types were measuring the same thing. The final magnetic BBA underestimated the polystyrene BBA values for almost all cytokines, but the overall cytokine profile was similar for both assay types. The optimized magnetic BBA showed expected variation for 27 cytokines within a given subject's tear sample. This variation is an improvement from initial magnetic BBAs that showed cytokines levels to be either all high or all low within a single tear sample.

In studies by previous students [35], ELISA, the gold standard, produced cytokine levels near zero when the polystyrene BBA readings were near zero. Therefore, a matrix effect was almost certainly occurring with the magnetic BBA because it had a positive cytokine reading when detection by the polystyrene BBA was near zero. Based on the direct correlation between the IL-8 and IP-10 ELISAs and their corresponding polystyrene BBA results, the polystyrene BBA was producing more reliable and valid results than the magnetic BBA.

Overall, the polystyrene BBA appears to be a better assay for tears. This study resulted in a failure to meet the original objective of producing magnetic BBA results equivalent to polystyrene BBA results. Given the comprehensive series of assays conducted, it appears that the magnetic bead assay in its current format is not compatible with tear cytokine assays.

CONCLUSION

The main goal of this project (Specific Aim 1) was to define a 27-Plex magnetic BBA for NS tears that provided equivalent results to a previously validated polystyrene BBA. In particular, it was crucial to consider the ability of the assay to quantify tear cytokine levels in the smallest possible NS tear sample. The reasons for differences in assay results required systematic investigation in order to achieve this goal.

In its initial form, the magnetic BBA appeared to be subject to systematic interference. This manifested as a tendency for cytokine levels to be all high or all low for a given subject. Therefore, if Subject 1 produced low levels of IL-1β, this subject would also produce low levels of most other cytokines.

The first magnetic BBA investigation determined if non antibody-based factors were contributing to the interference (Specific Aim 2). The plate type (plastic vs. filter), plate washing system (manual vs. automatic), and the Luminex system itself were tested to determine their contribution to the interference effect. Study of tear matrix/buffer/bead interactions was next. The study involved eight different buffers. For any buffer that showed sufficiently promising results, linearity of dilution studies were used to determine if the goal of high assay sensitivity could be realized.

In a series of direct comparison 27-Plex polystyrene and magnetic BBAs, TAB ultimately emerged as the most promising buffer. Both assay types used matched NS and

STIM tear samples. Linearity of dilution compared assay sensitivity and ability to detect cytokines in sub-5µL tear samples. Spike-recovery experiments investigated tear/buffer matrix effects and bead-matrix interactions.

Ultimately, a combination of Teknova assay buffer and filter plates eliminated the systematic interference initially seen in the magnetic BBA. However, linearity of dilution revealed that the magnetic BBA was not as sensitive as the polystyrene BBA. Spike-recovery experiments demonstrated a residual matrix effect in the magnetic BBA that was more substantial than observed with the polystyrene BBA.

Other investigators claimed that the antibodies used in the magnetic bead version of the Bio-Rad 27-Plex cytokine assay are not the same as in the polystyrene version. Given that the antibody sets used in each assay are proprietary, it is impossible to test this theory. Bio-Rad claims that the two assays are equivalent, but will not specifically state that the antibody sets are identical or otherwise. As the outcome of this study, the magnetic bead version of the Bio-Rad 27-Plex cytokine assay cannot be recommended as a substitute for the polystyrene bead version. This laboratory will therefore pursue other assay formats for tear cytokine analysis.

FUTURE STUDIES

Exploration of polystyrene BBA kits from manufacturers other than Bio-Rad may give results comparable to previously validated polystyrene BBA data. Use of other polystyrene kits appears necessary for future tear assays.

Researchers have put reducing agents in sputum samples, which have a similar cytokines composition as tears, to prevent chemokines and cytokines from binding to carrier proteins. Inaccurate cytokine readings can result from the binding to carrier proteins. Adding reducing agents may have a negative effect by interfering with the binding of secondary antibody to cytokines. One of these reducing agents, dithiothreitol (DTT), may be used in the future with tear samples to determine if it will reduce non-specific binding and changes in cytokine levels [47].

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APPENDIX A

INSTITUTIONAL REVIEW BOARD APPROVAL

THE UNIVERSITY OF ALABAMA AT BIRMINGHAM
Institutional Review Board for Human Use

September 19, 2011

MEMORANDUM

FROM: Cari Oliver
Assistant Director, OIRB

RE: Thesis Entitled: Tear Component Interference in Cytometric Bead-Based Biomarker Assays Using Magnetic Beads
Larezia Williams

Based on Data from Expedited Protocol X100704004 – Assay of Multiple Inflammatory Biomarkers for Use in Predictive Modeling of Ocular Surface Disease States

To Whom It May Concern:

The Office of the IRB (OIRB) has been notified that UAB student, Larezia Williams worked on the above-referenced protocol as part of the requirements for her master's thesis project.

The IRB was notified of this project prior to her beginning this work; however, she was listed as an "other investigator" on the application; therefore, her name does not appear on the original approval form. The UAB IRB cannot supply an alternate approval at this time but was aware of her work when the project started.

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The University of
Alabama at Birmingham
Mailing Address:
AB 470
1530 3RD AVE S
BIRMINGHAM AL 35294-0104

UAB

INFORMED CONSENT

irb

TITLE OF RESEARCH:	**Assay of multiple inflammatory biomarkers for use in predictive modeling of ocular surface disease states**
IRB PROTOCOL:	**X100704004**
INVESTIGATOR:	Dr. Roderick Fullard
OTHER INVESTIGATORS:	Dr. Keshia Elder, Dr. John Bradley, Dr. Tammy Than, Larezia Williams, Pearl Shin, Nicole Guyette, My Tho Karin Tran.
SPONSOR:	Eyesight Foundation of Alabama

Explanation of Procedures

We are asking you to take part in a research study. One hundred and twenty-five (125) participants will complete the study. Both dry eye patients and patients without dry eye are eligible for the study. This research study has two goals. The first is to investigate a new method to analyze proteins, called cytokines, in the tear fluid. Cytokines are associated with inflammation and can provide important information about the severity of dry eye. This new method of analyzing tear cytokines may allow us to use of smaller tear volumes than are currently required. This is important because many dry eye patients are only able to provide very small amounts of tear fluid. The second goal is to collect cells from the conjunctiva, a membrane covering the white part of the eye. The amount of a protein called ribonucleic acid (RNA) extracted from these cells will be used to determine if we can apply two different techniques to measure the production (expression) of cytokines at the surface of the eye.

We will be enrolling both dry eye patients and patients without dry eye for the study. During your first study visit, we will determine if you are suitable for this study based on your medical history. You will be asked to complete a brief survey to determine whether you have dry eye symptoms or not. Next, we will evaluate the health of the front of your eyes, with the same viewing techniques that are used in routine eye exams. Based on your responses to the questions about your health history, your responses on the survey, and the evaluation of the health of the front of your eyes, we will determine if you are eligible to continue. If so, we will perform two different procedures on your eyes, tear collection and conjunctival impression cytology. These may both be done in one visit or may be divided over two or more visits.

For tear collection, two different types of tear sample will be collected. The first will be a non-stimulated tear sample collected using a small polished collection tube placed gently near the lower lid close to the white portion of your eye. The second type of tear sample will be a stimulated tear sample. A cotton-tipped applicator will be gently inserted into your nose to stimulate the sneeze/tear reflex. This causes reflex tears to be produced. Tears will then be collected using a micropipette. These tear collection procedures are not uncomfortable or painful when applied properly and there is minimal risk involved.

For conjunctival impression cytology, a numbing eye drop will be placed in one eye. A small disc of filter paper will be gently pressed on the conjunctiva, the membrane covering the white

Participant's Initials _____

part of the front of your eye, then removed. Some surface cells will adhere to the filter paper disc. This procedure will be repeated for a maximum of eight disc placements. The procedure may be repeated for the other eye. The cells will be studied to determine the quantity of RNA collected from the front surface of your eye. After cell collection, the front surface of your eyes will be evaluated again and transient (temporary) dyes will be placed into the eye to assist with the evaluation.

Risks and Discomforts

Tear collection is usually pain free; however, minimal discomfort may occur if the collection tube accidentally contacts the eye or eyelid. Very rarely a minor abrasion to the front of the eye may occur. If an abrasion does occur, clinical facilities are located within the building for evaluation and treatment.

Collecting cells from the front of the eye is pain-free; however, minimal temporary discomfort may occur in some patients after the numbing drop wears off. Some patients may experience some temporary redness of the white parts of their eyes after the completion of the procedure. If a complication does occur, clinical facilities are located within the building for evaluation and treatment.

Temporary dyes used for viewing the front of your eye may cause minor stinging for some people that usually lasts no more than a few seconds.

Benefits

You will not benefit directly from taking part in this study. However, this study may help us better understand how to diagnose and treat inflammation of the eye in the future.

Alternatives

An alternative to this procedure would be not to participate in the study.

Confidentiality

Information obtained about you for this study will be kept private to the extent allowed by law. However, research information that identifies you may be shared with the UAB Institutional Review Board (IRB) and others who are responsible for ensuring compliance with laws and regulations related to research, including the Office for Human Research Protections (OHRP). Data from this study will be maintained confidentially, secured on the 4th floor of the School of Optometry building. The results of the study may be published for scientific purposes. However, your identity will not be given out.

Refusal or Withdrawal without Penalty

Your taking part in this study is your choice. There will be no penalty if you decide not to be in the study. If you decide not to be in the study, you will not lose any benefits you are otherwise owed. You are free to withdraw from this research study at any time. Your choice to leave the study will not affect your relationship with this institution.

Participant's Initials _____

You may be removed from the study without your consent if the study doctor decides it is not in the best interest of your health, or if you are not following the study rules.

What if you are a UAB Student? You may choose not to be in the study or you may withdraw from the study (stop) at any time before it is over. This will not affect your standing or grades at UAB. You will not be offered or receive any special consideration if you take part in this research.

What if you are a UAB Employee? Taking part in this research is not a part of your UAB duties, and refusing will not affect your job or relationship with UAB. You will not be offered or receive any special job-related consideration if you take part in this research.

Cost of Participation

There will be no cost to you from taking part in this study. All exams related to this study will be provided to you at no cost during the study period.

Payment for Participation in Research

You will receive a prepaid gift card for each of your study visits. Payment will be made at the completion of each study visit. At the first visit, if you do not qualify after completing the initial assessment (history, questionnaire and assessment of the health of the eye), you will be paid $10. If you do qualify to continue, you will be paid $10 for the initial assessments plus an additional $10 for each hour (or part thereof if a full hour is not completed) of tear collection. You will be paid $15 for the conjunctival impression cytology cell collection procedure. If you withdraw from the study, you will be compensated for the completed study visits.

Payment for Research-Related Injuries

UAB and the Eyesight Foundation of Alabama have not provided for any payment if you are harmed as a result of taking part in this study. If such harm occurs, treatment will be provided. However, this treatment will not be provided free of charge.

Significant New Findings

You will be told by Dr. Fullard or Dr. Elder if new information becomes available that might affect your choice to stay in the study.

Incidental Findings

You will be told by Dr. Fullard or Dr. Elder if there are any incidental health findings during the study. In the event of incidental health findings, the nature of the findings and recommended course of action, for example referral to an eye care practitioner or physician, will be explained to you.

Participant's Initials _____

Questions

If you have any questions, concerns, or complaints about the research or a research- related injury including available treatments, Dr. Roderick Fullard, Dr. John Bradley, or Dr. Keshia Elder, would be glad to address them. Dr. Fullard's number is (205) 934-6746. Dr. Bradley may be reached at (205) 934-9079 or after hours at (205) 201-9348. Dr. Elder's number is (205) 934-1227 and also be reached after hours at (205) 529-1015.

If you have questions about your rights as a research participant, or concerns or complaints about the research, you may contact Ms. Sheila Moore. Ms. Moore is the Director of the Office of the Institutional Review Board for Human Use (OIRB). Ms. Moore may be reached at (205) 934-3789 or 1-800-822-8816. If calling the toll-free number, press the option for "all other calls" or for an operator/attendant and ask for extension 4-3789. Regular hours for the Office of the IRB are 8:00 a.m. to 5:00 p.m. CT, Monday through Friday. You may also call this number in the event the research staff cannot be reached or you wish to talk to someone else.

Legal Rights

You are not waiving any of your legal rights by signing this informed consent document.

Signatures

Your signature below indicates that you agree to participate in this study. You will receive a copy of this signed document.

Signature of Participant Date

Signature of person obtaining consent Date

Signature of Witness Date

Signature of Investigator Date

Participant's Initials _____

University of Alabama at Birmingham
AUTHORIZATION FOR USE/DISCLOSURE OF HEALTH INFORMATION FOR RESEARCH

What is the purpose of this form? You are being asked to sign this form so that UAB may use and release your health information for research. Participation in research is voluntary. If you choose to participate in the research, you must sign this form so that your health information may be used for the research.

Participant Name:_____
Research Protocol: Assay of multiple inflammatory biomarkers for use in predictive modeling of ocular surface disease states

UAB IRB Protocol Number:
Principal Investigator: Roderick J. Fullard
Sponsor: Eyesight Foundation of Alabama

What health information do the researchers want to use? All medical information and personal identifiers including past, present, and future history, examinations, laboratory results, imaging studies and reports and treatments of whatever kind related to or collected for use in the research protocol.

Why do the researchers want my health information? The researchers want to use your health information as part of the research protocol listed above and described to you in the Informed Consent document.

Who will disclose, use and/or receive my health information? The physicians, nurses and staff working on the research protocol (whether at UAB or elsewhere); other operating units of UAB, HSF, UAB Highlands, The Children's Hospital of Alabama, Callahan Eye Foundation Hospital and the Jefferson County Department of Public Health, as necessary for their operations; the IRB and its staff; the sponsor of the research and its employees; and outside regulatory agencies, such as the Food and Drug Administration.

How will my health information be protected once it is given to others? Your health information that is given to the study sponsor will remain private to the extent possible, even though the study sponsor is not required to follow the federal privacy laws. However, once your information is given to other organizations that are not required to follow federal privacy laws, we cannot assure that the information will remain protected.

How long will this Authorization last? Your authorization for the uses and disclosures described in this Authorization does not have an expiration date.

Can I cancel the Authorization? You may cancel this Authorization at any time by notifying the Director of the IRB, in writing, referencing the Research Protocol and IRB Protocol Number. If you cancel this Authorization, the study doctor and staff will not use any new health information for research. However, researchers may continue to use the health information that was provided before you cancelled your authorization.

Can I see my health information? You have a right to request to see your health information. However, to ensure the scientific integrity of the research, you will not be able to review the research information until after the research protocol has been completed.

Signature of participant:_____ Date:_____
or participant's legally authorized representative:_____ Date:_____
Printed Name of participant's representative:_____
Relationship to the participant:_____
