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Human tear analysis with miniaturized multiplex cytokine assay on "wall-less" 96-well plate

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Purpose: Tears are a particularly limited body fluid and commonly used in the diagnosis of patients who have ocular diseases. A popular method for analysis of ocular inflammation in tears uses Luminex® bead multiplex technology to generate valuable multiple cytokine profile outputs with 25–50 µl tear sample volume. We propose a method for measuring tear cytokines with 5 µl tear sample volume and 80% reduced Luminex reagents compared to previous protocols.
Methods: Using human tears pooled from 1,000 participants, the DA-Bead-based method running at 5–20 µl volume, using manual pipetting, in conjunction with a magnetic Luminex cytokine (four-plex) panel assay in a 96-well format was performed and validated for tumor necrosis factor (TNF)-α, interferon (IFN)-γ, interleukin (IL)-1β, and IL-6.
Results: Upon use of the DA-Bead method at the 5 µl volume with cytokine standards, the concentrations of each of the four cytokines were found to be linear over a range of 3.5–4 log pg/ml with an intra-assay coefficient of variation (CV) ≤5%, inter-assay %CV ≤10%, and accuracy within the 70–130% range. Upon use of a 5 µl healthy pooled tear sample, cytokine concentrations were detected with a precision intra-assay %CV < 20% for IL-6, IFN-γ, or TNF-α or 30.37% with IL-1β. The inter-assay %CV with tears was ≤20.84% for all cytokines. Tear volumes run at 5 µl on DA-Bead produced a similar cytokine expression profile at a 1-month interval and were highly correlated with the larger 10 µl–based tear sample volume cytokine profile with $R^2 = 0.98$.
Conclusions: DA-Bead assay is highly sensitive and reproducible and has a performance profile that is potentially suitable for use in standard clinical scenarios. Considering the use of as little as 5 µl of assay beads and 5 µl sample, this is also likely to reduce the assay cost significantly and ease diagnosis of patients with ocular diseases.

Tear samples are increasingly being used for the diagnosis of various ocular surface disease states such as diabetic retinopathy, allergy, and dry eye [1,2]. Although enzyme-linked immunosorbent assay (ELISA) is commonly used for single analyte detection, many promising technologies today offer multiplex analyte detection from a single sample and help to maximize the analysis [3,4].

Different procedures are currently in place with patients for tear fluid recovery: Schirmer's test, capillary tube, or minisponge. Schirmer's test strips and capillary tubes have been shown to be reliable methods for tear collection and analysis with multiple cytokines [5,6]. These procedures are minimally invasive compared to procedures that collect impressions or brush cytologies.

Many studies have demonstrated the presence of cytokines in tears. Elevated levels of inflammatory cytokines in tears have been described as associated with various ocular surface diseases [3,7]. Recently, the standard operating instructions for evaluation of four tear cytokines was published, including recommendations for storage, transport, and performance of the immunoassays [6].

Luminex® technology (Luminex Corporation, Austin, TX), a form of bead-based sandwich indirect immunofluorescent assay, is one of the most established multiplex bead array methodologies for measuring multiplex cytokines in biologic applications with high sensitivity [6,8-14]. However, all Luminex assays are constrained by a minimum sample volume usage per well of 25 to 50 µl in a microtiter plate format. One major concern with the Luminex requirement and tear fluid in general is the smaller sample volume available and the low concentrations of analytes in the tear matrix compared to other biologic matrices such as plasma or urine. In the current procedure used in Luminex assays, tears are usually diluted and measured to minimum repeated levels, once or twice. Another common practice in certain scientific studies to circumvent the small volume of obtainable tears is the use of pooled tears; however, such methods are not appropriate for personalized medicine in which an individual patient's tear sample must be analyzed. From this perspective, there is an unmet need for an assay that handles small volumes. Quantitative proteomic methods such as isobaric tag

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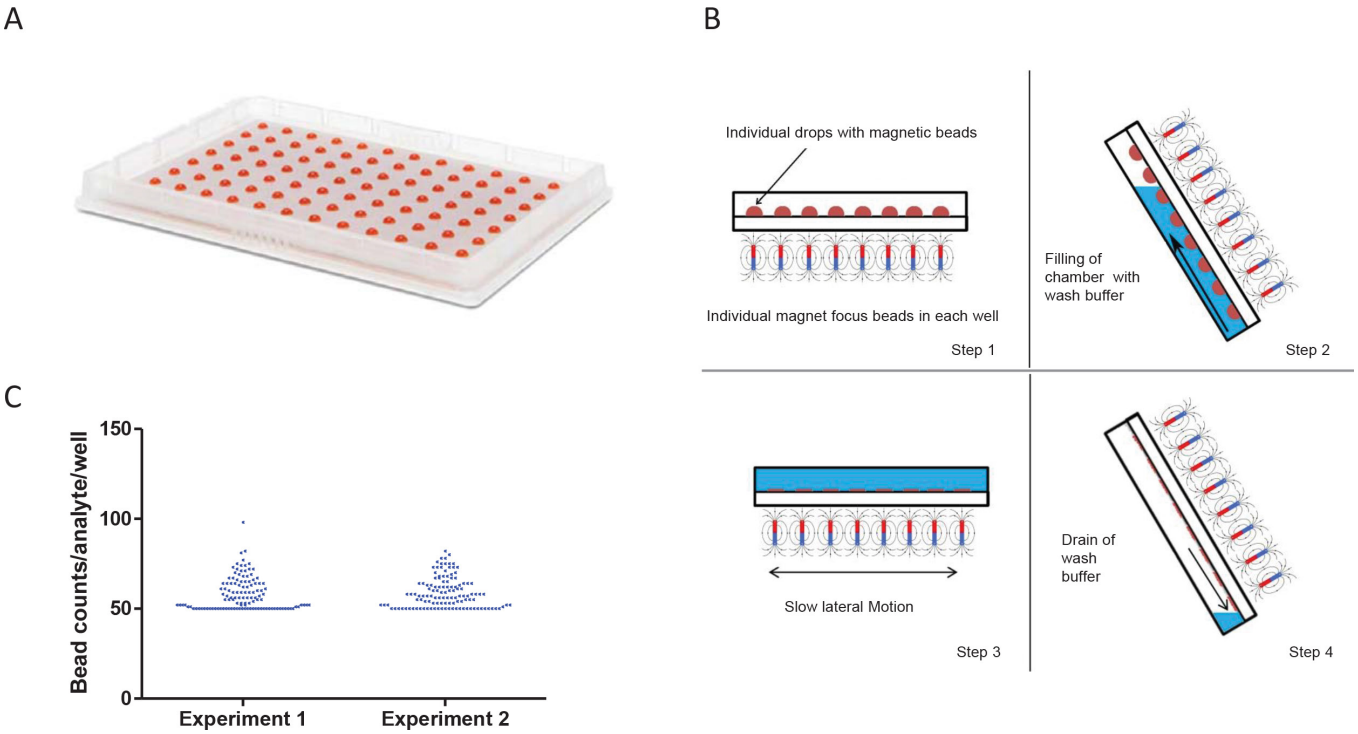

Figure 1. Overview of DropArray Technology. **A**: DA-Bead 96-well photograph. The plate follows 96-well microtiter plate standard specifications and accommodates drops of 5 µl up to 20 µl in hydrophilic well space surrounded by hydrophobic polytetrafluoroethylene (PTFE) space. **B**: Washing principle of DA-Bead with Curiox LT-MX washer. DA-Bead plate is placed in Washer LT-MX and sealed. Each drop assay is in line with an individual magnet, and focusing is performed for 30 s (Step 1). DA-Bead is rotated counter-clockwise to 120°, and washing buffer fills the plate from the bottom to the top (Step 2). DA-Bead is returned to the horizontal position. The rinsing chamber is then returned to a horizontal position and undergoes low velocity lateral shaking for 10 s at a speed of 20 rpm which is equivalent to a lateral shear force of 70.2 mm/s (Step 3). DA-Bead is rotated counter-clockwise to 120°, and the washing buffer is drained, producing a dry plate ready for the next reagent dispensing (Step 4). **C**: Bead count performance of experiments presented in this study on DA-Bead. Each dot represents the count for one analyte in a well. All bead counts are ≥50. Beads available per analyte in DA-Bead are reduced by 80% compared to conventional methods.

for relative and absolute quantitation with 1 µl tear volume offer feasibility in small volumes but suffer from a long process of sample preparation, time acquisition, and analysis/sample and are thus constraining for ease of diagnosis [15].

We present a novel Luminex assay approach developed on a new platform DA-Bead that circumvents the tear volume limitations of current Luminex-based assays while maintaining a similar process assay workflow. DA-Bead is based on DropArray technology, a wall-less plate that can miniaturize cell-based/biologic assays at up to 90% reduced scale [16-19]. DA-Bead (Curiox Biosystems, Singapore) is a wall-less plate defined with 96 circular wells on a polytetrafluoroethylene (PTFE) resin-coated polymer plastic and follows conventional microtiter plate specifications (Figure 1A). Each circular well is rendered hydrophilic through a combination of post-production process and plasma treatment. Each circular well can accommodate a drop between 5 and 20 µl volume. Drops remain confined to the hydrophilic circular well due to the surrounding presence of the hydrophobic resin. To provide optimum evaporation control during long incubation, an anti-evaporation lid is used on top of DA-Bead. An anti-evaporation lid is composed of a lid with an anchored medical-grade sponge material that absorbs PBS. Upon use of an anti-evaporation lid on top of a DA-Bead plate, high atmospheric humidity is established and provides evaporation control.

To perform uniform washing of the DA-Bead 96 wells during the Luminex magnetic-based assay, the DA-Bead plate is washed with the fully automated station LT-MX (Curiox Biosystems) that uses magnetic force, gravity, and pumps for the addition and removal of wash buffer (Figure 1B). DA-Bead is sealed in a washing station to form a rinsing chamber. After an initial 30 s magnetic bead focusing, the rinsing chamber is rotated counter-clockwise to 120°, and 80 ml of washing buffer is introduced through an inlet at the bottom of the chamber. The rinsing chamber is then returned to a horizontal

position and undergoes low velocity lateral shaking for 10 s at a speed of 20 rpm which is equivalent to a lateral shear force of 70.2mm/s. A second addition of 30 ml of washing is performed with the chamber rotated counter-clockwise to 30°. Finally, the chamber is rotated counter-clockwise to 120°, and the wash buffer is drained. More background on the theoretical principle of the Curiox washer with the wall-less plate can be found elsewhere [16-19].

In this report, we present a novel DA-Bead method for performing a Luminex-based magnetic assay and use as an example a multiplex measurement of tumor necrosis factor (TNF)-α, interferon (IFN)-γ, interleukin (IL)-1β, and IL-6. We present application of this method with the use of a tear sample volume of only 5 µl and a reduction of Milliplex-based kit reagent usage by 80%.

METHODS

Human tear samples: This study adhered to the Tenets of the Declaration of Helsinki. Human tear samples are based on a previous approved study [15] from 1,000 healthy subjects collected using the Schirmer tear test strips. After tear collection, the Schirmer strips were stored in −80 °C. Tear proteins from the Schirmer strips were extracted with PBS 1X (Na_2HPO_4 8.1 mm, KH_2PO_4 1.5 mm, NaCl 136.9 mM, KCl 2.7 mM, pH 7.3), and pooled in a single sample. Ethical approval (2009/910/A) for this study was obtained from the centralized institutional review board of SingHealth, and informed written consent was obtained from each patient.

Milliplex workflow with DA-Bead: To use the Milliplex kit (EMD Millipore Corporation, Billerica, MA) HCYTOMAG-60K with the DA-Bead 96-well plate, a reduction of 80% volume compared to the kit's recommendation was used. The beads, matrix, standard, sample, detection antibodies, and streptavidin-phycoerythrin volume were scaled down to 5 µl usage/well for each reagent instead of the 25 µl originally recommended. Bead content was reduced proportionally by 80% providing approximately 400 beads/analyte/well on DA-Bead with 5 µl volume compared to 2,000 beads/analyte/well on the conventional microtiter plate with 25 µl volume. The manual multichannel pipettes 1–10 µl range was used during all steps of this assay requiring 5–10 µl volume addition steps. The DA-Bead well surface was initially blocked with 1that 10 µl PBS with 1% bovine serum albumin (BSA) for 30 min at room temperature. DA-Bead was then washed in LT-MX washer once with wash buffer. PBS 0.1%, BSA 0.05%, and Tween-20 as an acting washing buffer were used during all washing steps of this assay. DA-Bead was then used with 5 µl bead addition followed by the 5 µl Milliplex assay buffer acting as the matrix. The 5 µl standard, controls, or tear sample was added last with a pipette mix of 5X. DA-Bead was then placed on an analog MicroPlate Genie Shaker (Scientific Industries Inc., Bohemia, NY) for 5 s at an intensity scale of 4. During incubation, DA-Bead was used with an anti-evaporation lid on top and was secured in a secondary opaque square sealed container prepared with 100 ml PBS distributed over eight side C-fold towels (Kimberly-Clarck, Irving, TX). A square sealed container provides a secondary control for minimal evaporation and provides high atmospheric humidity. The container was subsequently placed on an NB-101M orbital shaker (N-Biotek, Bucheon, South Korea) at 200 rpm (0.67 ×g) overnight at 4 °C. DA-Bead was then washed in LT-MX washer 3X with wash buffer. Then 5 µl detection antibodies/well was added. DA-Bead was then placed on an analog MicroPlate Genie Shaker for 5 s at an intensity scale of 4 and incubated again with the anti-evaporation lid in a square sealed container on the same shaker as described previously for 60 min at room temperature. An addition of 5 µl streptavidin-phycoerythrin/well was then performed. DA-Bead was then placed on an analog microplate genie shaker for 5 s at an intensity scale of 4 and incubated again with an anti-evaporation lid in a square sealed container on the same shaker as described previously for 30 min at room temperature. DA-Bead was then washed in LT-MX washer 3X with wash buffer. An addition of 5 µl Bio-Plex® (Bio-Rad, Hercules, CA) sheath fluid/well was then performed.

To accommodate a high concentration of beads at the reduced volume suitable for acquisition, two methods were used: The first method used a PCR plate with full-skirt PCR-96-FS-C (Axygen Scientific, Union City, CA). To transfer the bead content from DA-Bead to the PCR plate, DA-Bead was first placed on an analog microplate genie shaker for 5 s to release the beads from the plate surface. Addition by pipetting of 30 µl Bio-Plex sheath fluid/well and transfer of 30 µl from DA-Bead to PCR plate was performed 2X. The PCR plate was then shaken for 5 min at 800 rpm (1.07 ×g) at room temperature on a short orbital span thermomixer FP (Eppendorf, Hamburg, Germany). The Bio-Plex® 200 system (Bio-Rad) needle height was calibrated with the D6 well from the PCR plate with one microsphere added. The microsphere was from the kit CN-0015–01 (Luminex) and followed the Luminex guideline recommendations for calibration of V-bottom plates. The Bio-Plex 200 system was used following the recommended Milliplex kit guidelines at similar minimal 50 bead count requirements but with the following respective modifications: 40 µl injection volume and 60 s timeout. The second acquisition method used DA-Bead directly in the Luminex reader: After the last 3X LT-MX wash, 10 µl Bio-Plex sheath fluid was added in

each well instead of 5 µl. DA-Bead was then placed on an analog MicroPlate Genie Shaker for 5 s to release the beads from the plate surface. A 96-well reading block (Curiox) was stamped with 10 ml reading fluid (Curiox) and transferred on the DA-Bead plate. Only a small layer of reading fluid of approximately 1 ml was retained on the reading block after stamping in the 10 ml volume. The reading fluid layer acts as a seal between DA-Bead and the 96-well reading block. Then 35 µl Bio-Plex sheath fluid was added in each well and mixed 2X by pipetting. DA-Bead was then shaken for 5 min at 350 rpm (0.2 ×g) on the short orbital span ThermoMixer FP. The Bio-Plex® 200 system (Bio-Rad) needle height was calibrated with the D6 well from the DA-Bead plate with on a small disc added from the CN-0015-01 kit (Luminex). The Bio-Plex 200 system was used following the recommended Milliplex kit guidelines at similar minimal 50 bead count requirements but with the following modifications: 30 µl injection volume and 60 s timeout.

Milliplex assay analysis: The Milliplex kit HCYTOMAG-60K precustomized with IL-1β, IL-6, TNF-α, and IFN-γ analytes was used. The multiplex cytokine standard mixture from Milliplex HTH17–8014 was serially diluted with assay buffer fourfold on seven points. The diluted standard were assayed in two independent experiments with duplicate measurements. Precision was evaluated with the coefficient of variation (CV). The intra-assay %CV was generated from the mean of the %CV of two different concentrations of one experiment. Inter-assay %CV was generated across two different concentrations (second and fifth serial dilution) of two independent experiments. Accuracy is represented by the percentage recovery for each standard concentration; acceptable recovery is within 70–130% (Bio-Rad Principles of Curve Fitting for Multiplex Sandwich Immunoassays, Rev B). A reliable detection range was provided by Bio-Plex Manager™ software (Bio-Rad) with the lower limit of quantitation (LLOQ) and the upper limit of quantitation (ULOQ). The LLOQ-ULOQ defines a quantitative range where values can be estimated within an accuracy of 80–120% recovery and precision below 20% intra-assay %CV. Sensitivity is represented by the limit of detection (LOD) obtained by recalculating the concentration from 2 standard deviations of the background added to the average of the background reading. To establish batch to batch repeatability between experiments 1 and 2, two quality controls of known concentration ranges, as provided by the manufacturers, were measured in duplicate: QC1, a standard of known concentration within the lower concentration range (80–200 pg/ml), and QC2, a standard of known concentration within the higher concentration range (400–1,100 pg/ml). A concentration observed within the expected range was considered 100% recovery.

RESULTS

The DA-Bead miniaturized format offers the opportunity to design a Luminex assay with 80% fewer reagents than the conventional Milliplex protocol. DA-Bead is designed to use approximately 400 beads available per analyte with 5 µl bead usage compared to approximately 2,000 beads per analyte with 25 µl usage in a conventional Luminex assay. The bead counts for all analytes and all wells of experiments presented in this study with DA-Bead were ≥50 (Figure 1C) and satisfied conventional Milliplex bead count requirements despite this decrease in reagents. To investigate the performance of DA-Bead in the Milliplex assay, two independent experiments were performed with serial dilution of standards with expected known concentrations each with duplicate measurements. Five-parameter logistic standard curve fits were obtained with Bio-Plex Manager software (Figure 2). Fitting probability was in the range of 0.52 to 0.81, and the residual variance was calculated to range from 0.2 to 0.39 according to the analyte type. The best curve parameters were obtained with IL-1β. As shown in Table 1, the reliable range of detection (LLOQ-ULOQ) obtained for each cytokine covered 3.5 to 4 log range of the concentration depending on the analyte type. The measured concentration of the standard correlated well with the expected standard concentration ($R^2 \geq 0.99$). Precision analysis on DA-Bead showed an intra-assay %CV as low as 0.7% (IL-1β) up to a maximum level of 5.15% (IL-1β) depending on the cytokine and the experiments. The inter-assay CV% between both experiments was as low as 6% (TNF-α) up to the highest level of 9.71% (IL-6). The sensitivity of DA-Bead based on the LOD was 1.5 pg/ml for IL-6, 0.78 pg/ml for TNF-α, 1.19 pg/ml for IL-1β, and 2.66 pg/ml for IFN-γ. The Milliplex guidelines reported sensitivity as the minimum detectable concentration with 2 standard deviations were 1.3 pg/ml for IL-6, 1.1 pg/ml for TNF-α, 1 pg/ml for IL-1β, and 1.1 pg/ml for IFN-γ. We thus inferred that using DA-Bead achieved an almost equivalent expected sensitivity to the Milliplex expected sensitivity for IL-6 and IL-1β and slightly improved sensitivity for TNF-α but slightly lower sensitivity for IFN-γ..

The mean of the cytokine concentration, standard deviation of the mean, and percentage recovery of the standards in assay buffer are shown in Table 2. Recoveries at each diluting level were in the 90% to 110% range. Only one exception occurred with the highest concentration standard of IL1-β showing a higher variance with a decrease to 81% recovery, a phenomenon in line with the saturation of the curve approaching the ULOQ..

To assess reproducibility from experiment to experiment, QC1 and QC2 were used. IL-6 and TNF-α were measured

within the expected range, indicating 100% recovery as shown in Table 3. QC1 for IL-1β and IFN-γ showed higher recovery up to 132% for IL1-β or 118% for IFN-γ. The maximum %CV observed was 15% with TNF-α. These results are in line with the quality control criteria from the Milliplex guidelines.

To establish DA-Bead assay reliability with tear samples, we used pooled tears prepared from 1,000 healthy patients (Table 4). Aliquots of the same batch of pooled tears were used at a 1-month interval between two experiments. In both experiments, only IL-6 reached a detectable level within the reliable LLOQ-ULOQ range. IL-1β, IFN-γ, and TNF-α required an extrapolation beyond the ULOQ-LLOQ range due to the low cytokine expression in healthy tears. The intra-assay %CV observed was below 25% for all analytes with the exception of IL-1β with 30.3%. IL-1β was undetectable in the first experiment. The elevation in the IL-1β intra-assay %CV can be rationalized by the measurement close to the LOD level (Table 1). The inter-assay %CV based on both experiments performed was below 21% for IL-6, TNF-α, and IFN-γ.

We then compared whether the cytokine quantity observed with 5 µl tears would be in good agreement with the larger amount of tear sample volume at 10 µl. The intra-assay %CV was particularly lower for TNF-α and IL-1β with

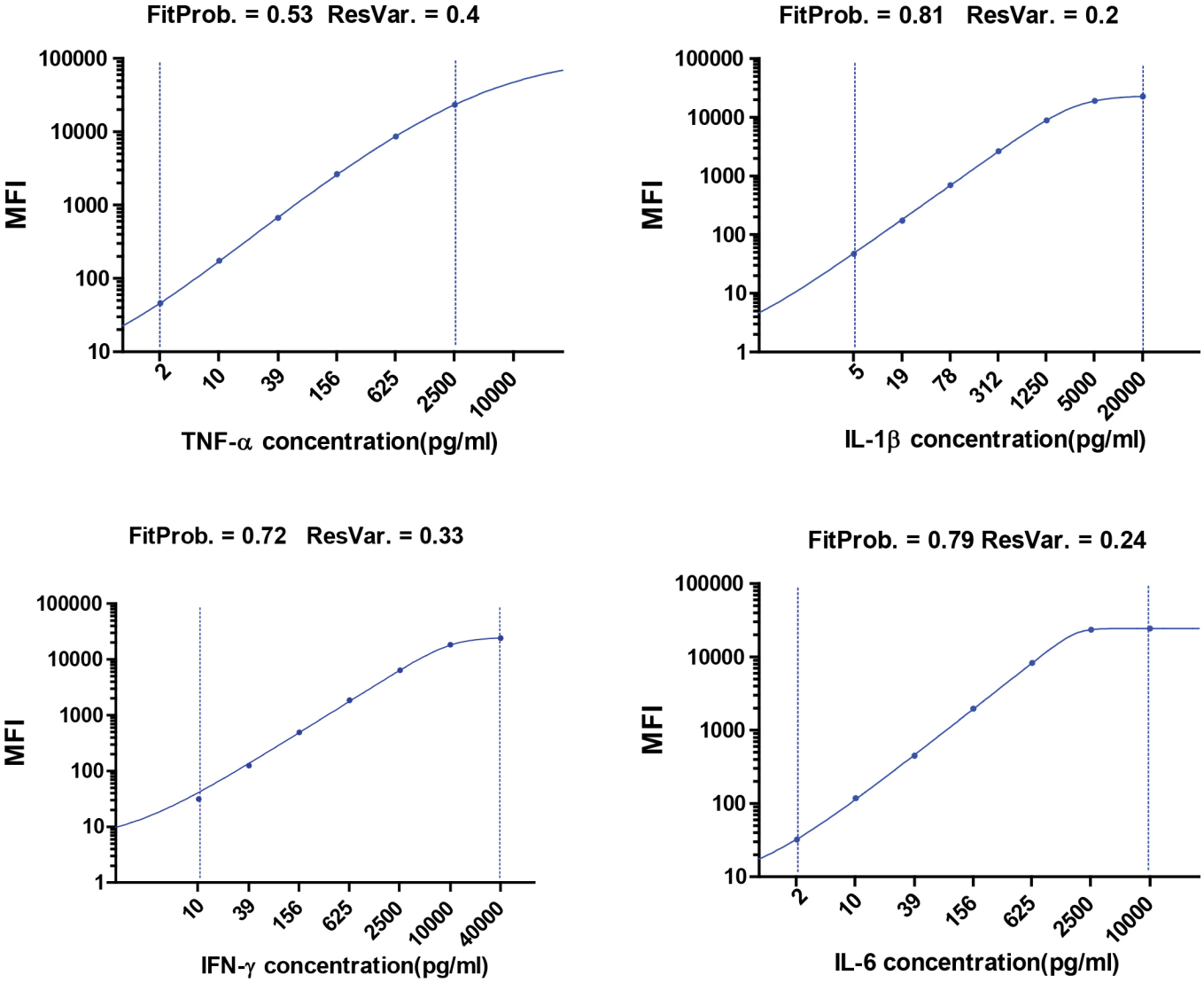

Figure 2. Five-parameter logistic curve of DA-Bead. Curves from experiment 2 are presented in this figure. The blue dotted line represents the lower limit of quantitation (LLOQ) and the upper limit of quantitation (ULOQ) calculated with Bio-Plex Manager software. FitProb and ResVar are the fitting probability and the residual variance, respectively, calculated by Bio-Plex Manager software for the indicated curve. The concentration and the median fluorescence intensity (MFI) scale are in the log range.

10 µl tears when compared to 5 µl (Table 4). This difference in precision can be expected in view of the larger amount of cytokine available for binding that provided a quantity further distant from the LOD when compared to the 5 µl volume (Table 1). The relative profile of cytokine expression between the two volumes tested was in good agreement with a correlation index of $R^2 = 0.98$ and confirmed the reliability of the DA-Bead cytokine profile measured in tears with 5 µl volume.

DISCUSSION

The data presented in this study demonstrated Luminex multiplex cytokine assay (IL-6, IL-1β, IFN-γ, and TNF-α) on DA-Bead with cytokine standards/quality controls with an intra-assay %CV ≤5% or an inter-assay %CV ≤10%, sensitivity to the pg/ml level, and accuracy within 70–130% accepted Luminex recovery guidelines. The DA-Bead performance is in line with the performance of a published study on conventional volumes [6]. The pooled tear sample from 1,000 healthy patients used at the 5 µl volume on DA-Bead showed detectable cytokine levels despite low expression and produced precision levels between 6.7% and 30.3% for the intra-assay %CV depending on the cytokine type. Precision between the two experiments with tears showed an inter-assay %CV range between 10.5% and 21% depending on the cytokine type. Quantification in tears performed at a 1-month interval between the two experiments on DA-Bead produced similar reportable quantities, and a similar relative profile was obtained between tears measured at 5 µl compared to 10 µl.

The range of the intra-assay %CV observed in our study with tears (6.7% to 30.3%) is similar to Wei et al.'s tear cytokine profile standard operating procedure study that reports an intra-assay %CV range between 9.6% and 37.56% with normal pooled tears with the same set of cytokine analytes as our study [6]. The IL-1β quantity measured in tears on DA-Bead can be discussed in view of multiple previous studies performed with bead multiplex technology. The IL-1β analyte was estimated at 1.49±0.45 pg/ml in the pooled tear sample we tested. An IL-1β single-plex bead study performed previously with healthy tears described low levels of IL-1β analyte below the LLOQ and reported sample levels at 0.62, 1.58, and 3.84 pg/ml [20]. A multiplex study with healthy tears extracted from sponges reported the IL-1β analyte level was undetectable [21]. Guyette et al. described IL-1β at 2.98±1.95 pg/ml in normal non-stimulated tears [22]. In a thyroid-associated ophthalmology study, the healthy control tears group collected with microcapillaries showed a low level of IL-1β analyte below the 2 pg/ml level [23]. Overall, the low level of IL-1β we detected with DA-Bead and bead multiplex ELISA in pooled healthy tears appears highly similar to the quantity range found in previous studies.

Luminex is a suspension array technology established to measure a multiplex cytokine profile with tear clinical samples in conventional microtiter plates [6,7,12,24-27]. Microtiter 96-well classical plates are currently constrained with a volume range of 25 µl to more than 200 µl, and this limits the Luminex assay if minimal sample volumes are not met. The 384-well microtiter plate assays are a suitable alternative for miniaturization of the Luminex assay below these volume ranges but require an investment in high-end automation and a Flexmap3D Luminex reader. The 384-well plates are not currently suitable for the common Luminex 200 or the MAGPIX Luminex reader. DA-Bead is a technology that can circumvent these limitations by adapting to an assay volume of 5–20µl in a conventional 96-well format for any Luminex reader with conventional pipettes and follows the same conventional Luminex workflow. Our results show that DA-Bead offers a suitable alternative to conventional plates for the analysis of tears. The volume of tears obtainable according to a standard operating process with capillary

Table 1. Summary of precision and accuracy determination with four cytokine standards.

Analytes	Experiment 1			Experiment 2			Inter-assay %CV	LOD pg/ml
	Detection range(LLOQ-ULOQ) pg/ml	Intra-Assay %CV	R^2	Detection range(LLOQ-ULOQ) pg/ml	Intra-Assay %CV	R^2		
IL-6	2.41–2501.65	5.04	1.00	2.40–9820–03	1.18	1.00	9.71	1.5
TNF-α	2.47–2496.87	4.58	1.00	2.43–2505.28	3.17	1.00	6.00	0.78
IL-1β	4.98–16024.06	0.7	0.99	4.93–17882.88	5.15	1.00	8.41	1.19
IFN-γ	10.06–37464.21	4.38	1.00	9.87–38786.96	3.82	1.00	8.44	2.66

Detection range was determined with Bio-Plex manager software. LOD was calculated as the mean +2 SD of the blank in both experiments. Intra-assay %CV: Intra-assay precision for each individual experiment. Inter-assay %CV: interassay precision of both experiments. R^2 was calculated by correlating measured concentration to expected concentration of standards.

Table 2. Recovery assays of cytokine standards diluted in assay buffer.

IFN-γ				IL-1β				IL-6				TNF-α			
Input (pg/ml)	Mean ± SD (pg/ml)	% Recovery	N	Input (pg/ml)	Mean ± SD (pg/ml)	% Recovery	N	Input (pg/ml)	Mean ± SD (pg/ml)	% Recovery	N	Input (pg/ml)	Mean ± SD (pg/ml)	% Recovery	N
40,000	40,439±10803.46	101	4	20,000	16,139.82±8396.28	81	3	10,000	NA	NA	4	10,000	NA	NA	4
10,000	10,300.97±366.62	103	4	5000	5443.81±613.75	109	4	2500	2493.63±25.96	100	4	2500	2474.81±8.53	99	4
2500	2412.21±42.22	96	4	1250	1217.79±37.70	97	4	625	624.17±1.11	100	4	625	623.55±23.19	100	4
625	652.84±95.61	104	4	312.5	316.70±24.52	101	4	156.3	157.44±15.18	101	4	156.3	156.99±8.83	100	4
156.3	157.93±9.08	101	4	78.13	79.07±2.57	101	4	39.06	38.33±1.89	98	4	39.06	39.02±2.45	100	4
39.06	37.44±3.22	96	4	19.53	19.06±0.43	98	4	9.77	9.97±0.95	102	4	9.77	9.75±0.92	100	4
9.77	9.96±0.61	102	4	4.88	4.95±0.12	101	4	2.44	2.40±0.45	98	4	2.44	2.44±0.18	100	4

NA: Not available, values beyond upper limit of quantitation range Mean and Standard deviation (SD) is calculated based on two experiments, each with duplicate measurements.

Table 3. Representative quality controls of four cytokines diluted in assay buffer.

Analytes	IL-1β			IL-6			IFN-γ			TNF-α		
	Experiment 1			Experiment 1			Experiment 1			Experiment 1		
ID	expected pg/ml	Recovery % [2+]	% CV	expected pg/ml	Recovery % [2+]	% CV	expected pg/ml	Recovery % [2+]	% CV	expected pg/ml	Recovery % [2+]	% CV
QC1[+]	88–182	132	6.9	96–199	100	5	105–214	118	3	94–208	100	15
QC2[+]	485–1008	107	3.2	532–1105	100	7	566–1176	100	3	483–1003	100	5.1
	Experiment 2			Experiment 2			Experiment 2			Experiment 2		
QC1[+]	88–182	112	2.4	96–199	100	6	105–214	100	0	94–208	100	4.7
QC2[+]	485–1008	107	9.4	532–1105	100	5	566–1176	101	1	483–1003	100	4.3

[+]QC1, quality control at lower concentration ranges; QC2, quality control at higher concentration ranges.[2+] Measurement that was within the expected ranges was considered as 100% recovery. For the measurement higher than the upper ranges of the expected, the percent recovery is higher than 100%.

Table 4. Level of four cytokines in 1000 patients pooled tear sample at 5 or 10 ml volume.

Analytes	Experiment 1 (5 µl) [#]			Experiment 2 (5 µl) [#]				Experiment 3 (10 µl) [##]		
	Mean±SD (pg/ml)	Intra-assay %CV	N	Mean±SD (pg/ml)	Intra-assay %CV	N	Inter-assay %CV	Mean±SD (pg/ml)	Intra-assay %CV	N
IL-6	12.93±-1.42	19.58	3	9.19±0.86	9.41	3	20.84	15.81±0.45	2.86	2
TNF-α	1.03±-0.13	12.73	3	1.47±0.27	18.14	3	10.47	2.47±0.29	12	2
IL-1β	NA[+]	NA[+]	0[+]	1.49±0.45	30.37	2[2+]	NA	2.4±0.08	3.23	2
IFN-γ	5.53±-0.98	17.62	3	5.87±0.39	6.78	3	12.04	9.23±0.7	7.58	2

[#]5 µl tear sample/5 µl beads reaction condition[##] 10 µl tear sample/5 µl beads reaction condition. [+]IL-1β below detectable level. . [2+]IL-1β with one well out of three wells below detectable level. Linear Correlation between results of 5 µl (Experiment 2) and 10 µl tears (Experiment 3) indicate R^2=0.98

tubes is 4–5 µl and is usually diluted to 50 µl to fulfill enough volume for single- or duplicate-well analysis with a Luminex assay kit. The Schirmer strips are normally applied to the patients' inferior fornix for 5 min and are commonly extracted with various volumes of buffers up to approximately 100 µl volume. In the context of dry eye disease, it is possible that the amount of wetting of the strip observed and therefore the tear proteins obtained, may be low. This would benefit from a lower sample volume of 5 µl instead of the current requirement of 25 µl. The minimal sample usage with DA-Bead can also increase the number of replicates for improvement in the consistency of diagnosis or for the remaining tear sample to be analyzed by other diagnostic tests. In addition to these improvements, Luminex reagents usage on DA-Bead are reduced by 80% and can bring the diagnostic cost of one patient eye by Luminex to about the US$9 range compared to the US$43 range for conventional methods (Table 5). From an eye clinic perspective and the patient perspective, DA-Bead can make a tear cytokine diagnostic process more affordable without incurring more time or a different learning process for the current operating staff since the workflow is similar to the conventional workflow. Use of DA-Bead with low tear volume samples could also turn out to be valuable for use in clinical trials of new therapies in ocular diseases.

In conclusion, profiling of tears cytokine with the DA-Bead plate follows the expected performance of conventional plates and offers a similar outcome as for the 5 µl tear volume instead of the 25 µl required conventionally. Use of DA-Bead can help to assess critical cytokine information

Table 5. Analysis of approximate reagent cost per sample with conventional and DA-Bead method.

Number of standards	7
Number of Blanks	1
Number of quality controls	2
Replicates for non experimental samples	2
Total wells for non samples	20
Number of patient samples	25
No of patient well replicates	3
Total wells for patient samples	75
Total number of wells used	95
Cost per plate of one conventional Luminex(4 cytokines) plate method	1083 US$ [#]
Cost per plate of Luminex reagents(4 cytokines) using DA-Bead method	216 US$ [#]
Cost per patient (Conventional Luminex; A)	43 US$ [##]
Cost per patient (DA-Bead Luminex; B)	9 US$
Savings (A-B)	34 US$
%Savings	80%

[#]Based on Millipore information online with Milliplex HCYTOMAG-60K (4 cytokines) as of 2015/04/13.[##] Based on cost shared between patients on one plate defined with conditions as described.

for bridging the gap with ocular disease diagnosis and may benefit in the monitoring of new ocular surface therapies.

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REFERENCES

1. Enríquez-de-Salamanca A, Bonini S, Calonge M. Molecular and cellular biomarkers in dry eye disease and ocular allergy. Curr Opin Allergy Clin Immunol 2012; 12:523-33. [PMID: 22895048].
2. Hang H, Yuan S, Yang Q, Yuan D, Liu Q. Multiplex bead array assay of plasma cytokines in type 2 diabetes mellitus with diabetic retinopathy. Mol Vis 2014; 20:1137-45. [PMID: 25253986].
3. Hagan S, Tomlinson A. Tear fluid biomarker profiling: a review of multiplex bead analysis. Ocul Surf 2013; 11:219-35. [PMID: 24112226].
4. von Thun Und Hohenstein-Blaul N, Funke S, Grus FH. Tears as a source of biomarkers for ocular and systemic diseases. Exp Eye Res 2013; 117:126-37. [PMID: 23880526].
5. VanDerMeid KR, Su SP, Krenzer KL, Ward KW, Zhang JZ. A method to extract cytokines and matrix metalloproteinases from Schirmer strips and analyze using Luminex. Mol Vis 2011; 17:1056-63. [PMID: 21552500].
6. Wei Y, Gadaria-Rathod N, Epstein S, Asbell P. Tear cytokine profile as a noninvasive biomarker of inflammation for ocular surface diseases: standard operating procedures. Invest Ophthalmol Vis Sci 2013; 54:8327-36. [PMID: 24204044].
7. Lee SY, Han SJ, Nam SM, Yoon SC, Ahn JM, Kim TI, Kim EK, Seo KY. Analysis of tear cytokines and clinical correlations in Sjogren syndrome dry eye patients and non-Sjogren syndrome dry eye patients. Am J Ophthalmol 2013; 156:247-53. [PMID: 23752063].
8. Massingale ML, Li X, Vallabhajosyula M, Chen D, Wei Y, Asbell PA. Analysis of inflammatory cytokines in the tears of dry eye patients. Cornea 2009; 28:1023-7. [PMID: 19724208].
9. Narayanan S, Corrales RM, Farley W, McDermott AM, Pflugfelder SC. Interleukin-1 receptor-1-deficient mice show attenuated production of ocular surface inflammatory cytokines in experimental dry eye. Cornea 2008; 27:811-7. [PMID: 18650668].
10. Corrales RM, Villarreal A, Farley W, Stern ME, Li DQ, Pflugfelder SC. Strain-related cytokine profiles on the murine ocular surface in response to desiccating stress. Cornea 2007; 26:579-84. [PMID: 17525655].
11. Lam H, Bleiden L, de Paiva CS, Farley W, Stern ME, Pflugfelder SC. Tear cytokine profiles in dysfunctional tear syndrome. Am J Ophthalmol 2009; 147:198-205. [PMID: 18992869].
12. Enríquez-de-Salamanca A, Castellanos E, Stern ME, Fernandez I, Carreno E, Garcia-Vazquez C, Herreras JM, Calonge M. Tear cytokine and chemokine analysis and clinical correlations in evaporative-type dry eye disease. Mol Vis 2010; 16:862-73. [PMID: 20508732].
13. De Paiva CS, Hwang CS, Pitcher JD 3rd, Pangelinan SB, Rahimy E, Chen W, Yoon KC, Farley WJ, Niederkorn JY, Stern ME, Li DQ, Pflugfelder SC. Age-related T-cell cytokine profile parallels corneal disease severity in Sjogren's syndrome-like keratoconjunctivitis sicca in CD25KO mice. Rheumatology (Oxford) 2010; 49:246-58. [PMID: 20007286].
14. Carreño E, Enriquez-de-Salamanca A, Teson M, Garcia-Vazquez C, Stern ME, Whitcup SM, Calonge M. Cytokine and chemokine levels in tears from healthy subjects. Acta Ophthalmol (Copenh) 2010; 88:e250-8. [PMID: 20738261].
15. Tong L, Zhou XY, Jylha A, Aapola U, Liu DN, Koh SK, Tian D, Quah J, Uusitalo H, Beuerman RW, Zhou L. Quantitation of 47 human tear proteins using high resolution multiple reaction monitoring (HR-MRM) based-mass spectrometry. J Proteomics 2015; 115:36-48. [PMID: 25529431].
16. Ingle GS, Scales SJ. DropArray, a wall-less 96-well plate for uptake and immunofluorescence microscopy, confirms CD22 recycles. Traffic 2014; 15:255-72. [PMID: 24329939].
17. Kozak KR, Wang J, Lye M, Chuh J, Takkar R, Kim N, Lee H, Jeon NL, Lin K, Zhang C, Wong WL, DeForge LE. Microvolume wall-less immunoassays using patterned planar plates. Lab Chip 2013; 13:1342-50. [PMID: 23380873].
18. Le Guezennec X, Phong M, Nor L, Kim N. Miniaturization of mitotic index cell-based assay using “wall-less” plate technology. Assay Drug Dev Technol 2014; 12:129-35. [PMID: 24611478].
19. Quiñones GA, Moore TI, Nicholes K, Lee H, Kim S, Sun L, Jeon NL, Stephan JP. Application of a new wall-less plate technology to complex multistep cell-based investigations using suspension cells. Blood 2013; 121:e25-33. [PMID: 23255559].
20. Narayanan S, Miller WL, McDermott AM. Conjunctival cytokine expression in symptomatic moderate dry eye subjects. Invest Ophthalmol Vis Sci 2006; 47:2445-50. [PMID: 16723455].
21. Inic-Kanada A, Nussbaumer A, Montanaro J, Belij S, Schlacher S, Stein E, Bintner N, Merio M, Zlabinger GJ, Barisani-Asenbauer T. Comparison of ophthalmic sponges and extraction buffers for quantifying cytokine profiles in tears using Luminex technology. Mol Vis 2012; 18:2717-25. [PMID: 23233782].
22. Guyette N, Williams L, Tran MT, Than T, Bradley J, Kehinde L, Edwards C, Beasley M, Fullard R. Comparison of low-abundance biomarker levels in capillary-collected nonstimulated tears and washout tears of aqueous-deficient and normal patients. Invest Ophthalmol Vis Sci 2013; 54:3729-37. [PMID: 23611994].
23. Huang D, Xu N, Song Y, Wang P, Yang H. Inflammatory cytokine profiles in the tears of thyroid-associated

ophthalmopathy. Graefes Arch Clin Exp Ophthalmol 2012; 250:619-25. [PMID: 22124787].

24. Benito MJ, Gonzalez-Garcia MJ, Teson M, Garcia N, Fernandez I, Calonge M, Enriquez-de-Salamanca A. Intra- and inter-day variation of cytokines and chemokines in tears of healthy subjects. Exp Eye Res 2014; 120:43-9. [PMID: 24412438].

25. Huang D, Luo Q, Yang H, Mao Y. Changes of lacrimal gland and tear inflammatory cytokines in thyroid-associated ophthalmopathy. Invest Ophthalmol Vis Sci 2014; 55:4935-43. [PMID: 24994866].

26. Jun AS, Cope L, Speck C, Feng X, Lee S, Meng H, Hamad A, Chakravarti S. Subnormal cytokine profile in the tear fluid of keratoconus patients. PLoS ONE 2011; 6:e16437-[PMID: 21298010].

27. VanDerMeid KR, Su SP, Ward KW, Zhang JZ. Correlation of tear inflammatory cytokines and matrix metalloproteinases with four dry eye diagnostic tests. Invest Ophthalmol Vis Sci 2012; 53:1512-8. [PMID: 22323462].

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