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Accurate Multiplex Cytokine Assay Developed with VeraCode® Technology

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Introduction

Cytokines are low molecular weight hormone-like polypeptides that are secreted during the course of immunologic and inflammatory responses. They are important regulators of cell-mediated and humoral immune responses and their differential expression has been associated with a wide variety of immune disorders[1-4]. They function on a variety of cell types, having stimulatory or inhibitory effects on proliferation, differentiation, and maturation. Given this pleotropic effect, measuring the level of only a single cytokine in any biological system provides only partial information relevant to the response on a systematic level. Therefore, comprehensive tests for cytokine levels generally aim to measure the concentrations of a large set of cytokines to gain a better understanding of the underlying physiology.

The enzyme-linked immunosorbent assay (ELISA) is the most commonly used and reported method for the quantitation of secreted cytokines. However, ELISA can only detect one analyte per reaction in individual assay wells. This translates to high reagent costs, excessive technician time, and large sample volumes required to generate each result. The ability to detect and quantitate many cytokines simultaneously in the same sample via a robust multiplexed assay would reduce these costs and improve efficiency. Advantages of multiplex technology over conventional assay methods include simultaneous analyte detection, reduced reagent handling, high output rates of test results, and reduction of required sample and reagent volumes[5-8].

We have developed a multiplexed cytokine assay using VeraCode technology as the assay platform. The assay detection chemistry is similar to that of ELISA, but is adapted to take advantage of the multiplex and high-throughput capabilities of VeraCode technology. VeraCode technology platform uses two major components. The first is the VeraCode bead, a holographically encoded 28 × 240 micron silica cylinder. Because the beads are made of silica, their surfaces are ideal substrates for molecular assays. The bead surface can be functionalized with various biomolecules, including the carboxyl groups that were used to covalently attach specific antibodies in the experiments described below. The VeraCode beads are, in effect, a solid assay substrate with the advantageous kinetics and handling characteristics of a solution. The second component of the technology is the BeadXpress® Reader, a two-laser scanning CCD imager, which identifies individual beads and detects their assay reporter signals. The refraction-based holographic codes used to identify the inscribed code elements do not interfere with the fluorescence signals generated by the assay.

To create specific cytokine assays, capture antibodies were covalently linked to individual types of VeraCode Carboxyl Beads, which were then pooled to create multiplex assays. Samples, standards, or controls were incubated with the antibody-coated VeraCode bead pools, which capture the analyte from solution. After washing to remove unbound analyte, a biotin-conjugated secondary antibody was added. The conjugated secondary antibody was bound to the analyte to complete the "sandwich." The complex was then washed to remove unbound detection antibody and incubated with phycoerythrin reporter-conjugated streptavidin. Assays were then read out by simultaneous detection of the fluorescent reporter signal and the identifying code embedded in the VeraCode beads using the Illumina BeadXpress Reader.

The experiments performed and described below show that a 10-plex cytokine assay developed on the BeadXpress platform is a robust multiplex method to measure the concentrations of several cytokines in a single sample. Sensitivity, specificity, precision, dynamic range, and accuracy were measured and all metrics exhibited accurate results that, in combination, support a high-quality multiplex assay.

Materials and Methods

Cytokine Reagents

Human cytokine reagents were purchased from commercial sources (Table 1). DuoSet ELISA development kits containing capture/detection antibodies and recombinant protein standard for IFN-γ, IL-1b, IL-4, and IL-8, were purchased from R&D Systems (Minneapolis, MN). Cytokine matched-pair antibodies (capture/detection) along with recombinant protein standards for TNF-α, IL-5, and IL-6 were purchased from R&D Systems. Capture/detection antibodies along with recombinant protein standards for IL-2, IL-10, and IL-12 (p70), were purchased from BD Biosciences (Franklin Parks, NJ). Biotin-conjugated goat anti-mouse Ig antibody was purchased from Sigma (St. Louis, MO). Phycoerythrin-conjugated streptavidin was purchased from Invitrogen (Carlsbad, CA).

Additional Reagents

EDC (1-(3-dimethylaminopropyl)-3-ethyl-carbodiimide HCl) and Sulfo-NHS (N-Hydroxysulfosuccinimide) were purchased from Pierce (Rockford, IL). MES (2-(N-Morpholino)ethanesulfonic Acid), bovine serum albumin (98% minimum), and phosphate buffered saline (10x concentrate) were purchased from Sigma. RPMI 1640 was purchased from Invitrogen. Penicillin-Streptomycin was purchased from Cambrex BioSciences (Walkersville, MD). Pooled normal mouse serum, normal rat serum, normal human serum, whole blood (citrate), and normal plasma (EDTA, heparin, citrate) were purchased from Innovative Research (Southfield, MI). Tween-20 was purchased from EMD Chemicals (Gibbstown, NJ). Proclin 300 was purchased from Supleco (Bellefonte, PA). Kingfischer 96-well stripwell plates were purchased from Thermo Electron Corp (Vantaa, Finland), and round bottom 96-well microtiter plates were purchased from VWR. Wide-orifice pipette tips (200 µl) were purchased from Axygen Scientific (Union City, CA).

Table 1: Cytokine Reagents Used in the Development of a 10-Plex Cytokine Assay

Analyte	Vendor	Capture Ab	Standard	Detection Ab
INF-γ	R&D Systems	MAB2852	285-IF-100	BAF 285
TNF-α	R&D Systems	MAB610	210-TA	BAF 210
IL-1b	R&D Systems	MAB601	201-LB-005	BAF 201
IL-2	BD Biosciences	554424	554603	555040
IL-4	R&D Systems	MAB604	204-IL-010	BAF 204
IL-5	R&D Systems	MAB405	205-IL-010	BAM6051
IL-6	R&D Systems	MAB206	206-IL-010	BAF 206
IL-8	R&D Systems	MAB208	208-IL-010	BAF 208
IL-10	BD Biosciences	554705	554611	554499
IL-12 (p70)	BD Biosciences	555065	554613	554660

Covalent Attachment of Capture Antibodies to VeraCode Carboxylate Microbeads

Capture antibodies were immobilized to VeraCode Carboxyl Bead Sets (Illumina, San Diego, CA), using the cross linking reagents EDC and sulfo-NHS. A single tube containing ~25,000 carboxy-terminated VeraCode beads was washed three times with 0.1M MES, pH 4.5. As described in the supplied VeraCode Assay Guide[4], 50 mg each of EDC and sulfo-NHS were resuspended individually with 1.0 ml of 0.1M MES, pH 4.5. After removal of residual buffer, the carboxyl VeraCode beads were activated with the EDC and Sulfo-NHS solutions. First, 500 µl of the Sulfo-NHS solution was added to the VeraCode beads. The mixture was vortexed for 5 seconds and 500 µl of the EDC mixture added. This mixture was vortexed and incubated in a Vortemp 56 (Labnet International, Woodbridge, NJ) at 100 rpm for 60 minutes at ambient temperature. The beads were then centrifuged for 5 seconds and washed twice with 0.1M MES, pH 4.5 to remove excess crosslinking reagents. The capture antibodies were diluted to 100 µg/ml in 0.1M MES, pH 4.5 buffer, and added to individually activated tubes of VeraCode beads. It is important to have the protein free of other proteins or amine-based salts that can inhibit the coupling reaction of the antibody. Each capture antibody listed in Table 1 was coupled to the beads via the available amino groups for 60 minutes at 100 rpm at ambient temperature in a Vortemp. The coupled VeraCode beads were washed twice with 0.2% Tween-20/PBS (PBST) to remove unbound antibodies. After two additional washes with 1M NaCl, the VeraCode beads were incubated for 1 hour at room temperature with 1.0 ml of NaCl buffer in the Vortemp as before. Following two washes with 1% BSA/PBS, the beads were blocked in 1.0 ml of 1% BSA/PBS for one hour at room temperature with 1.0 ml of 1% BSA/PBS in the Vortemp as described. The VeraCode beads were washed two additional times with 1% BSA/PBS and resuspended in 1.0 ml 0.05% Proclin 300/1% BSA/PBS. Beads were stored at 4°C until use.

Quantitation of VeraCode Beads After Antibody Immobilization

VeraCode beads are easily counted visually under a standard laboratory microscope. Individual tubes of immobilized VeraCode beads were resuspended by vortexing the tube for 30 seconds and pipetting up and down 10 times using a P200 pipette with a wide orifice tip set to deliver 50 µl. A 50 µl aliquot of bead slurry was diluted into 0.95 ml of PBST in a 1.5 ml Eppendorf tube. The bead slurry was mixed by pipetting 10 times. Three aliquots of 50 µl were spotted on a microscope slide. Resuspension, dilution, and spotting were repeated for each VeraCode bead type. Under low power (10×), the number of

Table 2: Microscopic Quantitation of Veracode Beads

Bead Set Code	Rep	beads / 50µl	Average	SD	% CV	Intermediate	Original Stock	% Retention
8210	1	57	54	3	6	1073	21,467	86
	2	51						
	3	53						
8260	1	58	57	4	6	1140	22,800	91
	2	60						
	3	53						
8216	1	52	54	2	3	1073	21,467	86
	2	54						
	3	55						

Table 3: Quantitation by Dry Mass

Bead Set Code	Process Step	Rep	Mass (mg)	AVG Mass (mg)	SD	% CV	Mass Difference (mg)	Conversion (µg/bead)*	% Retention**
8210	Pre-Immobili-zation	1	1,219	1,219	0.32	0.03	1.03	0.32	87.2
		2	1,219						
		3	1,219						
	Post-Immobili-zation	1	1,218	1,218	0.15	0.01			
		2	1,218						
		3	1,218						
8216	Pre-Immobili-zation	1	1,198	1,198	0.35	0.03	1.27	0.32	84.2
		2	1,198						
		3	1,197						
	Post-Immobili-zation	1	1,197	1,196	0.25	0.02			
		2	1,196						
		3	1,196						

*0.32 µg = 1 VeraCode bead **24,166 VeraCode beads / tube

beads in each of the 50 µl spots were counted manually. The average number of beads per slide (3 × 50 µl spots) was used to calculate the number of beads per ml using a dilution factor of 400 (Table 2). Percent retention was then calculated as the fraction of beads that were successfully labeled.

To validate the accuracy of the microscope counting method, we calculated the number of beads (% retention) after the immobilization process by an analytical method using dry mass measurement. Tubes of VeraCode beads were washed with 100% ethanol and dried in a SpeedVac for 15 minutes. The tare masses of the tubes were determined by weighing each in triplicate prior to the immobilization process. The tubes were carried through the immobilization process as described above, except that the final wash was with 100% ethanol. The beads were dried and weighed in triplicate. The results are shown in Table 3. Table 4 is a summary showing the consistency of the percent recoveries calculated by each of the two different measurement methods.

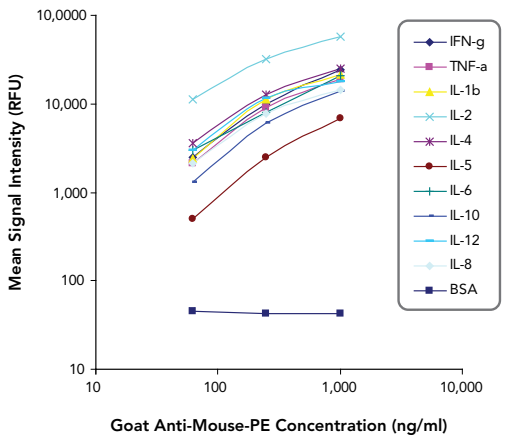
Efficiency of Antibody Coupling

The coupling efficiency of immobilized antibodies to the Carboxyl VeraCode beads can be examined by titration of a fluorescently labeled antibody. This method serves as a qualitative method for determining whether the antibody coupling was successful. For instance, a monoclonal capture antibody immobilized to a VeraCode bead can be detected with Phycoerythrin-labeled anti-Ig antibody. All types of cytokine-coupled beads were incubated with 50 µl serially diluted Phycoerythrin-labeled anti-Ig antibody in PBST buffer for 30 minutes at room temperature with agitation. The beads were washed three times with PBST, resuspended in 75 µl of PBST, and scanned in the BeadXpress Reader. Figure 1 shows the signal intensities generated after serial dilution of goat anti-mouse PE antibody in the 10-plex cytokine assay. BSA-coated VeraCode beads were used as a specificity control. A dose-dependent curve is observed, as expected for

Table 4: Comparison of Quantitation Methods

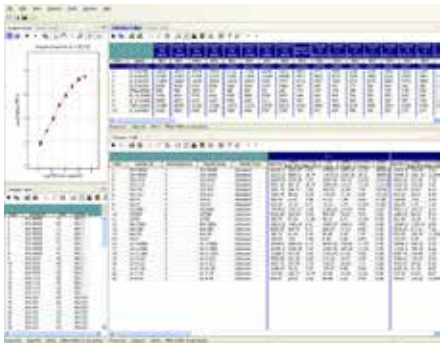
Bead Set Code	% Retention by Quantition Method	
	Microscope	Dry Mass
8210	86	87.2
8216	86	84.2

Figure 1: Titration of PE-Labeled Goat Anti-Mouse IG Antibody



Signal intensity of antibody-conjugated VeraCode Beads detected with PE-anti-Ig antibodies increases in a dose-dependent manner, unlike the negative control. The signal intensities for all bead types are significantly higher then the negative control. These results indicate that capture antibodies are successfully covalently bound to the beads.

Figure 2: Beadstudio Data Analysis Software Protein Module



[1]BeadStudio Data Analysis Software Protein Module collects all raw intensity data and produces summary measurements and calibration curves.

successfully coupled beads, and most assays exhibited greater than 1,000 RFU signal intensity when a minimum of 50 ng/ml labeled secondary antibody was used.

VeraCode Beads Kitted into 96-Well Microtiter Plates

Immobilized VeraCode beads were delivered into 96-well microtiter plates using a VeraCode Bead Kitting System according to manufacturer's instructions. The Bead Kitting System is designed to evenly distribute VeraCode Beads in all wells of a 96-well microtiter plate. First, 160 ml 30% Ethanol/PBS was dispensed into the Kitting System. A sufficient volume of VeraCode bead suspension was then added to yield an average of 15–20 VeraCode beads of each type per well. A 96-well plate adaptor and microtiter plate were then positioned onto the Kitting System. The whole assembly was shaken briefly, inverted, and allowed to stand for two minutes. The microtiter plate was washed twice with 0.05% Tween-20/PBS. The resulting prepared assay plates were sealed and stored at 4°C, or used immediately.

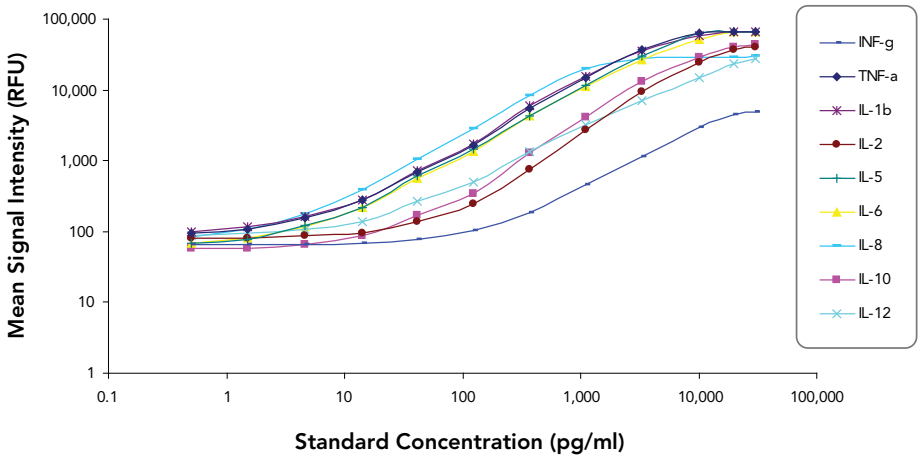
Cytokine Assay Buffers

Cytokine standards diluent (CSD) consisted of 0.1% Tween-20 in 1× PBS (pH 7.2) supplemented with a proprietary formulation of animal protein and serum. Proclin 300 (an anti-microbial preservative) was added to CSD at a final concentration of 0.05%. Cytokine reagent diluent (CRD) consisted of 1× PBS (pH 7.2), 0.1% BSA, 0.05% Tween-20, and 0.05% Proclin 300. Both diluent solutions were filtered through a 0.2 µm filter (Millipore, Bedford, MA) and stored at 4°C. Wash buffer consisted of 0.05% Tween-20 in PBS (pH 7.2).

Multiplex Cytokine Assay

The 10-plex multiplex cytokine assay was designed to measure the concentrations of each of the following analytes in a sample: INF-γ, TNF-α, Il-1b, IL-2, IL-4, IL-5, IL-6, IL-8, IL-10, and IL-12 (p70). A multiplex standard curve was prepared using 3-fold serial dilutions of a multiplex protein standard in CSD (Figure 3). Aliqots of 50 µl multiplex standard were added to wells containing VeraCode beads. Additionally, 50 µl of controls or samples was added to remaining wells. The VeraCode beads, standards, controls, and samples were incubated for one hour at ambient temperature with agitation on a plate shaker. Three washes with PBST were performed, consisting of the addition of 150 µl wash buffer, a quick spin of the microtiter plate (1500 rpm for 5 seconds), and aspiration of wash buffer using a 12-pin aspirator. The VeraCode beads were subsequently incubated with 50 µl multiplex detection cocktail for one hour at ambient temperature with agitation. The multiplex cocktail contained biotinylated antibodies specific for all 10 cytokine analytes at ~150 ng/ml. Beads were washed three additional times to remove non-specifically bound detection antibodies. Phycoerythrin-conjugated streptavidin assay reporter was incubated with beads for 30 minutes at a final concentration of 6.4 µg/ml diluted in PBST. To remove any non-specific labeling, an additional three washes with PBST were performed. The fluorescent signal intensity and bead identification codes of bound complexes were then detected in the BeadXpress Reader.

Figure 3: Multiplex Detection Range of 10-Plex Cytokine Standards in Serum Diluent



Signal intensities of each cytokine-bead type are plotted for all cytokine concentrations measured (0.5 pg/ml to 2500 pg/ml). All assays have at least a 3- to s4-log range of increasing in a dose response relationship. The high end of the dynamic range for each analyte in the 10-plex cytokine panel was estimated from the curve.

Table 5: Recovery of Standards in Different Dilution Matrices

	Concentration pg/ml	IL-1	IL-10	IL-5	IL-4	IL-2	INF-γ	IL-6	TNF-α	IL-8	IL-12 (p70)
Cytokine Standards Diluent (CSD)	2500	97	107	95	85	99	89	94	101	123	92
	1000	110	104	99	91	101	90	98	105	106	97
	500	98	94	93	91	89	96	96	94	100	100
	200	78	72	76	71	94	80	83	84	81	87
	100	63	99	68	55	61	54	69	78	65	77
	50	64	55	62	35	55	52	62	72	62	67
	25	54	37	51	59	47	56	52	61	53	57
	Average	81	81	78	70	78	74	79	85	84	82
	Range	54–110	37–104	51–99	35–91	47–101	52–96	52–98	61–105	53–106	57–100
Tissue Culture Medium	2500	125	93	89	97	99	83	94	99	98	90
	1250	95	103	95	97	97	89	95	107	92	93
	156	85	95	89	92	97	103	95	100	81	96
	20	103	95	96	93	108	81	128	112	91	104
	Average	102	97	92	95	100	89	103	104	91	96
	Range	85–125	93–109	89–95	92–97	97–108	81–103	95–128	99–112	81–92	90–104
Pooled Normal Human Serum	1000	105	104	93	55	104	101	125	101	63	83
	100	85	94	81	81	93	71	106	71	55	70
	50	95	109	92	154	109	78	119	78	59	76
	25	87	90	86	116	96	69	100	69	49	78
	Average	93	99	88	102	101	80	113	80	57	77
	Range	85–105	90–109	81–93	55–154	93–109	71–101	100–125	69–101	49–63	70–83

Instrumentation and Calculation

The BeadXpress Reader was used to scan the results of all plates. From each well, 5–30 beads for each analyte type were scanned, and the green fluorescence units were counted. Data from all such beads and their corresponding relative fluorescence unit (RFU) values were reported and used in downstream analysis. All standards and samples were measured at least in triplicate. Cytokine concentrations in samples were quantitated using the standard curves. The calibration curves for each analyte were calculated by the BeadStudio 3.0 Protein (PT) Module software (Figure 2) using a 5-parameter logistic regression (5 PL).

Results and Discussion

After creating a multiplex cytokine assay by adapting ELISA detection chemistry to the VeraCode technology, we wanted to test the performance of this assay in controlled experiments. Important performance metrics for multiplex protein detection assays are accuracy, sensitivity, specificity, precision, and reproducibility. We tested the 10-plex cytokine assay in experiments using various controls and known standards to qualify the assay performance on each of these metrics.

Table 6: LOD and Dynamic Range of Multiplex Cytokine Assay

Analyte	LOD (pg/ml)	Dynamic Range (pg/ml)
INF-γ	4.5	4.5–30,000*
TNF-α	0.5	0.5–30,000*
IL-1b	0.5	0.5–20,000
IL-2	0.5	0.5–30,000*
IL-4	14	0.5–30,000*
IL-5	0.5	0.5–20,000
IL-6	0.5	0.5–20,000
IL-8	4.5	4.5–5,500
IL-10	0.5	0.5–30,000*
IL-12 (p70)	0.5	0.5–30,000*

*30,000 pg/ml was the highest concentration tested

Assay Accuracy

To measure the 10-plex assay accuracy, recombinant protein standards of known concentration provided by the manufacturer were used in spike-in experiments. Cytokine standards diluent was spiked with eight different concentrations of all 10 cytokine standards and analyzed to determine the accuracy of the measured concentration value. Known standard concentrations were chosen in the linear range of the assay and within the concentrations represented in the standard curve. Tissue culture medium and pooled human serum were spiked with four different concentrations of multiplexed standard. Four replicates of each concentration were assayed on each plate. The spiked samples were quantitated using the standards curve and expressed as percent recovery (Table 5). At all but the lowest concentrations, the assays exhibited very high accuracy measurements.

Assay Sensitivity

The assay sensitivity, or limit of detection (LOD), for each cytokine, is defined as the corresponding concentration at three standard deviations above the mean fluorescence of at least eight replicates of the 0 pg/ml negative control (data not shown). The LOD ranged from 0.5 pg/ml (the lowest concentration measured for the standard concentration curve) to 14 pg/ml in one case (Table 6). Three-log to greater than four-log dynamic ranges for individual analytes are evident from the linear portion of the sigmoidal standard curves from the 10-plex assay (Figure 3).

Table 7: Specificity of Multiplex Cytokine Assay

Capture Ab on Bead	Protein Standard Applied (2500 pg/ml)									
	IL-1b	**IL-2**	**IL-4**	**IL-5**	**IL-6**	**IL-8**	**IL-10**	**IL-12 (p70)**	**TNF-α**	**INF-γ**
IL-1b	**8,416**	123	122	125	119	120	122	121	125	122
IL-2	123	**1,615**	122	125	118	120	123	121	122	126
IL-4	199	185	**2,258**	166	156	158	215	155	157	163
IL-5	152	151	150	**11,345**	142	245	141	191	145	148
IL-6	161	163	159	168	**5,392**	151	151	157	259	159
IL-8	185	148	129	187	142	**3,768**	183	196	128	146
IL-10	162	167	154	162	152	155	**2,970**	153	155	155
IL-12 (p70)	163	164	161	167	156	155	159	**1,821**	158	166
TNF-α	170	167	164	169	155	155	212	163	**6,376**	161
INF-γ	166	164	161	169	155	155	155	158	161	**5,320**
Bead Only	135	118	125	140	151	133	148	145	156	138

Table 8: Percision of Multiplex Cytokine Assay

	2500 pg/ml (n=8)	**500 pg/ml (n=8)**	**50 pg/ml (n=8)**	**Intra-Assay CV (n=24)**
Plate 1	8.0	5.3	6.0	**6.4**
Plate 2	5.8	4.7	4.2	**4.9**
Plate 3	5.9	5.2	4.4	**5.2**
Plate 4	7.5	16.3	7.5	**10.4**
Plate 5	7.0	9.6	7.1	**7.9**
Plate 6	9.9	8.9	4.9	**7.7**
Plate 7	8.5	5.2	5.1	**6.3**
Plate 8	8.4	5.5	5.9	**6.6**
Plate 9	14.8	11.1	6.2	**10.3**
Plate 10	8.0	5.3	6.0	**6.4**
Plate 11	5.8	4.7	4.2	**4.9**
Inter-Assay CV	**8 (n=88)**	**8 (n=88)**	**6 (n=88)**	**7 (n=264)**

Table 9: Linearity of 10-Plex Cytokine Assay

Dilution Factor	Linearity (Observed/Expected x 100%)									
	IL-2	IL-12	TNF-α	IL-1	IL-10	IL-6	IFN-g	IL-8	IL-4	IL-5
1:2.5	114	100	120	114	108	106	100	130	95	106
1:5	116	113	124	117	121	111	104	138	112	116
1:25	97	102	100	95	65	103	71	95	92	105
1:50	107	103	101	107	185	105	64	101	90	108
1:100	110	94	104	98	103	108	75	98	79	102
Avg	**109**	**103**	**110**	**106**	**116**	**106**	**83**	**113**	**94**	**108**

Assay Specificity

Specificity is essential in multiplex assays because the rate of non-specific interactions increases with increasing plexity and more complex reagent mixtures. Thus, high-quality multiplex assays must be highly specific to minimize false-positive results. To determine specificity in this 10-plex cytokine assay, individual cytokine standards at 2500 pg/ml in CSD were incubated with the 10-plex bead pool, according to the protocol previously described. Table 7 shows the specific signal of the individual analytes to their cognate beads and the intensities of non-specific off-target signals. Results are reported as mean RFU of eight replicates for each analyte. The specific signal falls on the diagonal axis (grey cells). Although the background signal levels varied on the different bead types, highly specific signals were observed only on the VeraCode beads with the specific on-target capture antibody. All off-target background intensities are similar to bead-only negative controls. The differences in RFU intensity for on-target signals observed for different standards are chiefly explained by the variable affinities of different capture antibodies for their cognate analyte. Across all analytes, the average on-target signal was more than 30 times greater than the average off-target background signal.

Assay Precision and Reproducibility

To analyze the precision and reproducibility of the multiplex cytokine assay, cytokine standards were spiked in normal human serum at final concentrations of 2500, 500, and 50 pg/ml. Eight replicates of each concentration were tested per plate. A total of 11 plates were run by five different operators over seven days at two sites. Plates were read on two different BeadXpress Readers. The coefficients of variation for data sets from each plate were calculated and are listed in Table 8. The intra-assay coefficient of variation (CV), a measure of the variation between all replicates within a plate, ranged from 4.9–10.4% (average 7%). The inter-assay CV, a measure of the variation of replicates between individual assay plates, ranged from 4–14% (average 8%). The average CV over all plates, days, and replicates was 8%.

Linearity and Dynamic Range

Interpolation of experimental results from an assay calibrated using standard concentration solutions is only valid over a range where assay output fluorescence is linearly related to input concentration. To examine the range over which this 10-plex cytokine assay yields linear results, the multiplex standard was spiked with pooled human serum as a source for cytokines. Serum (2,500 pg/ml) was serially diluted in a range of 1:2.5 to 1:100 in pooled human serum matrix and tested in triplicate using the 10-plex cytokine assay. The observed/expected (O/E) ratio was calculated by dividing the observed signal by the expected signal. The expected signal was defined as the observed signal of the next higher dilution, adjusted by the dilution factor (Table 9). The range of concentrations over which an assay exhibits O/E values of 80–120% is generally considered sufficiently linear to yield accurate results. All analyte assays were linear over most of the range of concentrations examined.

Empirical Standard Curve Testing

The cytokine quantitation in this multiplex assay provides absolute concentration values by relating readout RFU values to a standard curve. Thus, a high goodness of fit between data and the standard curve are essential to obtain accurate measurements. The goodness of fit was assessed by calculation from experiments using known analyte standards and spiked-in standards. The quality of the BeadStudio Protein Module curve fit was assessed by calculating the concentrations of the standards after regression. A recovery value between 70–130% is considered acceptable and is defined as observed/expected × 100%. Average standards recovery was about 100% (range 96–112%) for all analytes tested (Table 10). The consistency of data points and the standard curve confirms the accuracy of concentration determinations based on this 10-plex assay.

Conclusion

We have developed and validated a quantitative assay for the simultaneous concentration determination of 10 human cytokines using the innovative VeraCode technology and BeadXpress Reader. VeraCode technology offers several practical advantages for developing multiplex assays. VeraCode beads are digitally encoded with unique signatures, allowing multiplexed detection based on light scattering rather than fluorescence. VeraCode technology facilitates low- to mid-multiplex levels with convenient handling characteristics and quasi-solution phase kinetics, which substantially increases the speed of assays compared to solid phase. Beads are easily retained during wash steps and require no specialty plates or apparatus for handling. Additionally, Illumina has developed a Bead Kitting System that simplifies delivery of statistically consistent bead populations into individual wells of a 96-well plate. This device eliminates the need for manual pipetting of beads which can be a source of failures in other bead-based systems. Finally, the unique size and shape of the VeraCode beads facilitates their use in undiluted serum or whole blood and in other problematic samples where smaller beads may exhibit excessive loss.

Table 10: Empirical Standards Recovery as Expected From Standard Curve Calculation

	Standards Recovery (%)									
Concentration (pg/ml)	**IL-1**	**IL-10**	**IL-5**	**IL-4**	**IL-2**	**INF-γ**	**IL-6**	**TNF-α**	**IL-8**	**IL-12 (p70)**
10,000	104	108	100	100	103	101	101	109	41	92
3,333	94	100	99	98	97	98	97	100	111	110
1,111	113	103	106	105	106	106	106	114	102	106
370	95	95	91	95	98	94	96	88	96	94
123	105	105	110	110	102	102	106	105	112	102
41	86	95	92	77	90	98	90	90	92	92
14	103	109	111	201	147	113	107	106	105	111
4.5	93	91	92	-	65	85	89	95	82	100
1.5	131	108	108	-	132	105	119	117	133	98
0.5	93	100	99	-	105		94	90	91	-
Avg	**102**	**101**	**101**	**112**	**104**	**100**	**101**	**101**	**96**	**101**
Range	**93–113**	**91–109**	**92–111**	**77–110**	**65–147**	**85–113**	**89–119**	**88–117**	**41–133**	**92–111**

The findings described here show that the 10-plex cytokine assay based on VeraCode technology perform very highly on test experiments designed to measure sensitivity, specificity, reproducibility, and accuracy. Verifying the high performance of the multiplex assay under tightly controlled experiments is an indication that the assay is likely to perform well and provide accurate measurements in experimental conditions. The assay is sensitive enough to detect a wide range of cytokine concentrations with high signal-to-noise ratios, due in part to low cross-assay off-target interactions. The accuracy of all 10 multiplexed assays and robust reproducibility indicate this is a platform that will produce highly reliable measurements.

Furthermore, confirming that this cytokine multiplex assay exhibits high performance metrics indicates that the VeraCode technology is likely to provide a highly successful high-throughput platform for widespread use with other multiplex biomarker assays.

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