

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 disorders¹⁻⁴. They function on a variety of cell types, having stimulatory or inhibitory effects on proliferation, differentiation, and maturation. Given this pleiotropic 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 volumes⁵⁻⁸.

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). Kingfisher 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 μ l) 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 Guide⁴, 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 μ l of the Sulfo-NHS solution was added to the VeraCode beads. The mixture was vortexed for 5 seconds and 500 μ l 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 μ g/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 μ l. A 50 μ l 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 μ l were spotted on a microscope slide. Resuspension, dilution, and spotting were repeated for each VeraCode bead type. Under low power (10 \times), the number of

Table 2: Microscopic Quantitation of Veracode Beads

| Bead Set Code | Rep | beads / 50 μ l | 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 ($\mu\text{g}/\text{bead}$)* | % Retention** |
|---------------|---------------------|-----|-----------|---------------|------|------|----------------------|---|---------------|
| 8210 | Pre-Immobilization | 1 | 1,219 | 1,219 | 0.32 | 0.03 | 1.03 | 0.32 | 87.2 |
| | | 2 | 1,219 | | | | | | |
| | | 3 | 1,219 | | | | | | |
| | Post-Immobilization | 1 | 1,218 | 1,218 | 0.15 | 0.01 | | | |
| | | 2 | 1,218 | | | | | | |
| | | 3 | 1,218 | | | | | | |
| 8216 | Pre-Immobilization | 1 | 1,198 | 1,198 | 0.35 | 0.03 | 1.27 | 0.32 | 84.2 |
| | | 2 | 1,198 | | | | | | |
| | | 3 | 1,197 | | | | | | |
| | Post-Immobilization | 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 ($3 \times 50 \mu\text{l}$ 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.

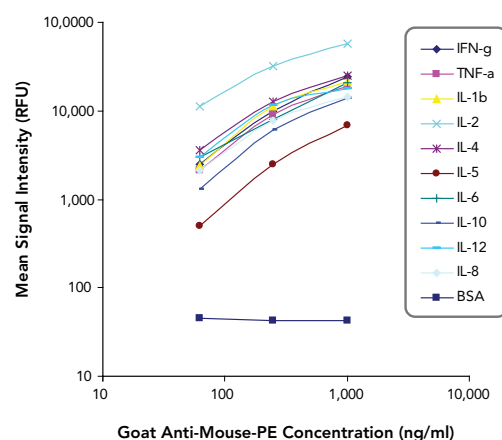
To validate the accuracy of the microscope counting method, we calculated the number of beads (% retention) after the immobilization process by an analytical method using dry mass measurement. Tubes of VeraCode beads were washed with 100% ethanol and dried in a SpeedVac for 15 minutes. The tare masses 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

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

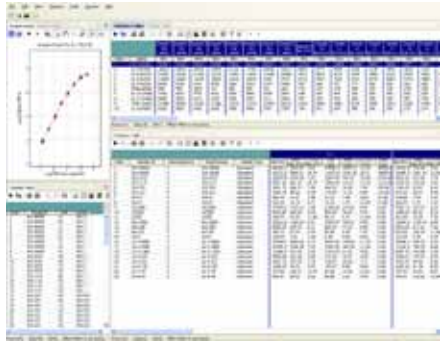


Signal intensity of antibody-conjugated VeraCode Beads detected with PE-anti-Ig antibodies increases in a dose-dependent manner, unlike the negative control. The signal intensities for all bead types are significantly higher than the negative control. These results indicate that capture antibodies are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

Table 4: Comparison of Quantitation Methods

| Bead Set Code | % Retention by Quantitation Method | |
|---------------|------------------------------------|----------|
| | Microscope | Dry Mass |
| 8210 | 86 | 87.2 |
| 8216 | 86 | 84.2 |

Figure 2: BeadStudio Data Analysis Software Protein Module



¹BeadStudio 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.

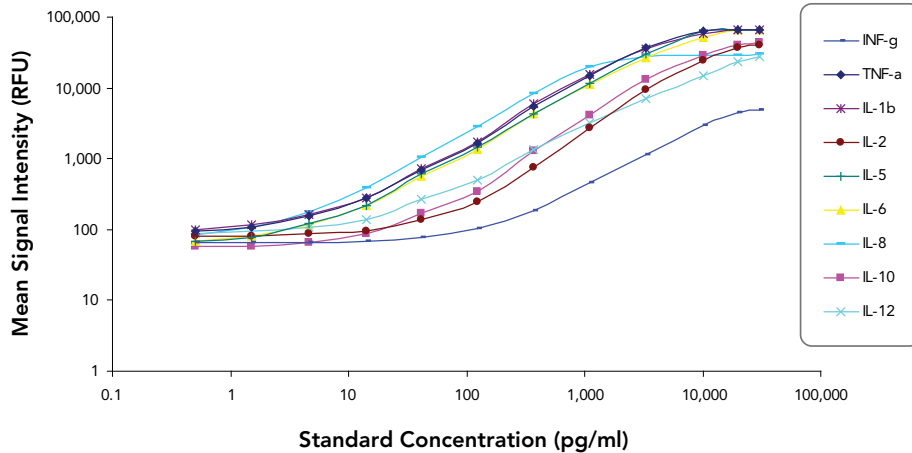
Cytokine Assay Buffers

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). Aliquots 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 4-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: Precision 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 \times 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

| Concentration (pg/ml) | Standards Recovery (%) | | | | | | | | | |
|--------------------------|------------------------|---------------|---------------|---------------|---------------|---------------|---------------|---------------|---------------|----------------|
| | 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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