

Multiplex Cytokine Immunoassay with Barcoded Magnetic Beads (BMB)

To create specific cytokine assays, capture antibodies were covalently linked to individual types of carboxyl BMB. Different BMB-probes were then pooled into a tube or microwell to create a mix for multiplex assays. The sample was incubated with the mixed BMB pools. Biotin-conjugated secondary antibody was added to complete the “sandwich” reaction. The complex was washed to remove unbound secondary antibody, and then incubated with SA-PE (fluorophores). The microplate was placed in a BioCode-1000 Analyzer for barcode decoding/identification and fluorescence detection.

A. Multiplex Cytokine Assay

Materials

Cytokine Reagents:

Human cytokine reagents containing capture/biotinylated secondary antibodies and recombinant protein standard for GM-CSF, IL-1 β , IL-6, and IL-8 were purchased from Invitrogen (CytoSet, 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), Tween-20 were purchased from Sigma (St. Louis, MO), bovine serum albumin (98% minimum) were purchased from Equitech-Bio (Kerrville, TX), and nuclease free H₂O and phosphate buffered saline (10x concentrate) were purchased from Ambion (Foster City, CA). Proclin 300 was purchased from Supleco (Bellefonte, PA). And, phycoerythrin-conjugated Streptavidin (SA-PE) was purchased from Invitrogen (Carlsbad, CA).

Covalent Attachment of Capture Antibodies to BMB

Capture antibodies were immobilized to carboxyl BMB sets with a two-step coupling procedure using the cross-linking reagents EDC and sulfo-NHS.

The first step: a single 2ml tube containing ~5,000 of one type carboxyl BMB were vortexed, washed once with 500 μ l nuclease free H₂O, and once with 500 μ l MES buffer 25mM, pH 6.0, quick spinned, and then BMB were collected with a magnetic stand. After removal of residual buffer, the BMB were activated by adding 200 μ l of the freshly prepared Sulfo-NHS solution (25mg/ml in MES buffer). The mixture was vortexed for five seconds and 200 μ l of the freshly prepared EDC solution (25mg/ml in MES buffer) was added. This mixture was vortexed and incubated in a Vortemp 56 (Labnet International, Woodbridge, NJ) at 280 rpm for one hour. The

beads were then washed twice with 500 μ l MES buffer with a magnetic stand to remove excess crosslinking reagents.

The second step: 200 μ l of capture antibodies (diluted in MES buffer, 25-50 μ g/ml) were added to individually activated tubes of BMB. Each capture antibody was coupled to the beads via the available amino groups for 2 hours, shaking at 280 rpm. The coupling reaction was blocked by incubating the BMB with 500 μ l Tris-HCl buffer 50mM, pH 7.4, for 15 min with rotation. The coupled BMB were washed once with wash buffer (PBS, 0.05% Tween-20, 1mM EDTA), and were blocked in 500 μ l of Assay Buffer (PBS, 0.5% BSA, 0.1% Tween-20, 0.5% ProClin) for one hour at room temperature with rotation. The BMB were then resuspended in 500 μ l assay buffer; the BMB number can be measured by the BioCode-1000 Analyzer. Individual BMB (~5,000 beads in 500 μ l buffer) were stored at 4°C until further use. Bead concentration is approximately 10 beads/ μ l in a 2 ml tube.

Multiplex Probes Stock Solution Preparation

The 4-plex multiplex cytokine (GM-CSF, IL-1 β , IL-6, and IL-8) assay was prepared by pooling the four bead types coupled with capture antibodies specific for GM-CSF, IL-1 β , IL-6, and IL-8, respectively, in a 2 ml tube. The number of needed beads depends on the total number of samples (microwells) to be tested. For example, if each bead type is determined to be 50 beads (should be > 20 beads), 4-plex, 10 microwells, the total number of beads in the stock solution should be > 50x 4 x10 = 2,000 beads; this is based on 200 beads per microwell. The typical bead concentration in stock solution is in the range of 2.5 - 50 beads/ μ l.

B. Multiplex Cytokine Assay

The washing process can be performed with either a magnetic stand or a filter plate. In the case of using a filter plate, pre-wet 96 filter plate wells (Millipore, Billerica, MA) with 100 μ l wash buffer (remove buffer using vacuum), and then with 100 μ l Assay Buffer. Aliquot 50 μ l (~ 200 beads) of multiplex BMB were added into a well. Then 50 μ l of sample or standard (2,000pg/ml) were mixed with BMB and incubated for one hour (RT, 700rpm). Multiplex biotinylated antibodies (50 μ l, pre-prepared according to the manufacture kits) were added, and incubated for an additional hour, and BMB were washed two times with 200 μ l wash buffer. Finally, SA-PE (150 μ l) (10 μ g/ml in assay buffer) was added in, and incubated for 30 minutes (RT, 700rpm), and then washed twice. The resulting BMB solutions were transferred to a flat bottomed 96-well clear microplate for optical detection. The fluorescence intensity and barcodes were then detected and recognized by BioCode-1000 Analyzer. The fluorescence intensity is determined based on a trimmed mean for every bead type.

BMB	Analyte added			
	GM-CSF	IL-1 β	IL-6	IL-8
GM-CSF	2110	10	15	18
IL-1 β	11	1126	16	9
IL-6	28	6	769	5
IL-8	42	8	46	3568

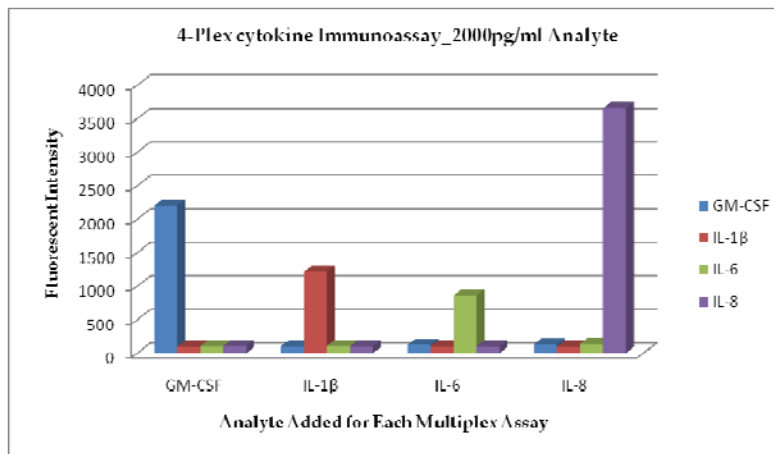


Fig. 1: Specificity – 4-plex immunoassay.

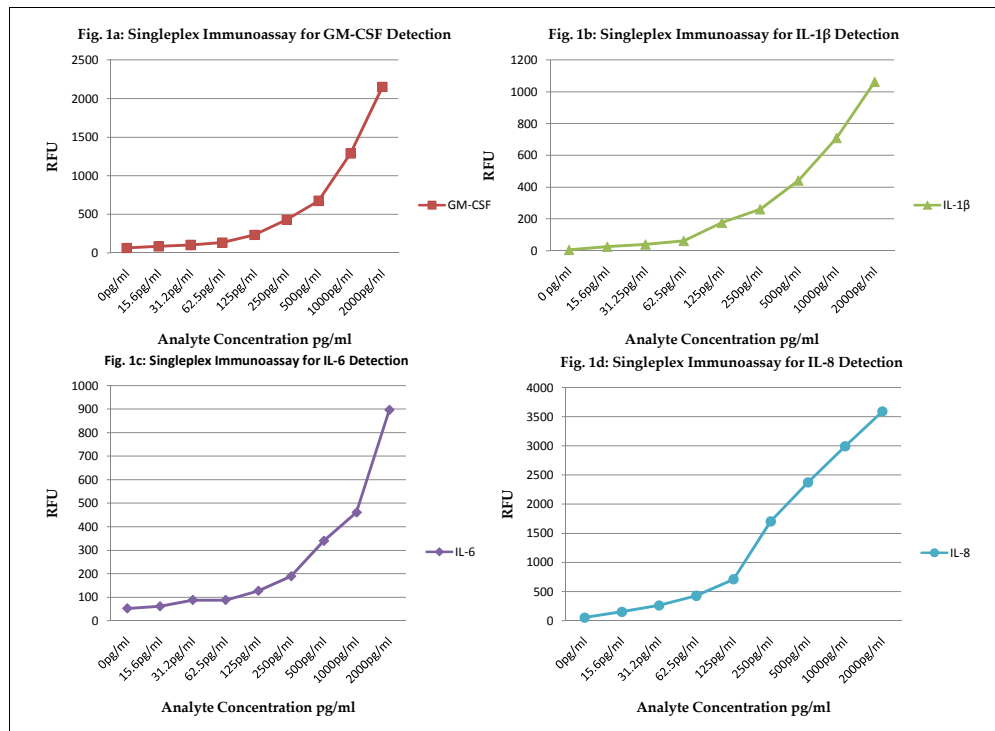


Fig. 2: Sensitivity – Single-plex immunoassay.