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Multiplex Immunoassays for the Detection of Extracellular and Intracellular Analytes Using Flow Cytometry

JOHN M. CASPER, Jennifer M. Storie, Jennifer V. Samoy, Eric B. Bertelsen and Michael C. Mullenix

Assay Designs Inc., 5777 Hines Drive, Ann Arbor MI 48108

INTRODUCTION

Assay Designs has launched a multiplex bead immunoassay platform (MultiBead™) which incorporates flow cytometry as the assay readout. The immunoassays are carried out on the surface of fluorophore labeled polystyrene beads. The beads themselves are internally labeled with precise amounts of a single fluorophore such that separate populations of the beads can be identified by the flow cytometer based on the fluorescence intensity. Capture antibodies are coated onto the surface of the beads. Separate immunoassays can be carried out simultaneously on each different bead type. In sandwich immunoassay formats, antigen in the sample is captured by the antibody on the bead and detected using biotinylated detector antibodies. Signal is generated in the assay through subsequent addition of streptavidin labeled with a second fluorophore which emits at a different wavelength from the fluorophore in the beads. Additional multiplexing is achieved by building different immunoassays on different size beads. The flow cytometer enables simultaneous identification of bead types by size and fluorescence intensity and provides a quantitative readout of the fluorescence generated through binding of the biotinylated detector antibodies to the captured antigen. In sandwich immunoassays, as antigen concentration increases signal also increases. The platform also enables the use of competitive immunoassays formats by incorporating a labeled form of the target antigen as the detection system. In competitive assays, antigen in the sample competes with a fluorophore labeled form of the antigen for binding to the capture antibody. In competitive immunoassays the intensity of the signal is inversely proportional to the amount of antigen in the sample. The multiplex assay platform has been validated for the detection of both intracellular and extracellular human targets. The extracellular targets are proteins and small molecules involved in inflammation and include 19 cytokines and 2 eicosanoids related to inflammation. The intracellular targets are all proteins related to the heat shock response and cell signaling. The MultiBead platform also includes innovative, flexible and easy to use software for data analysis. The software is easy to customize, enables end users to customize custom panels as well as add their own analytes. The software utilizes interpolation from point to point, 4pL or 5pL standard curve fitting to report back unknown values in a user customizable report.

MATERIALS AND METHODS

Experiments were performed in 96-well filter plates and analyzed with a BD FACSCalibur. Standard curve and sample concentrations were generated using Assay Designs MultiBead Analysis Software compatible for use with FCS and LMD files.

Basic Assay Protocol

- 25 µL of bead cocktail (supplied at 10X stock)
- 50 µL of standard or diluted sample
- 25 µL of PE-eicosanoid conjugate
- Shaken for one hour at room temperature, then washed 3X
- 100 µL of Cytokine Biotinylated Ab Cocktail (supplied at 10X stock)
- Shaken for 1 hour at room temperature, then washed 3X
- 100 µL of Streptavidin-PE conjugate (supplied at 10X stock)
- Shaken for 30 minutes at room temperature, then washed 3X and analyzed in flow cytometer

ASSAY FORMAT

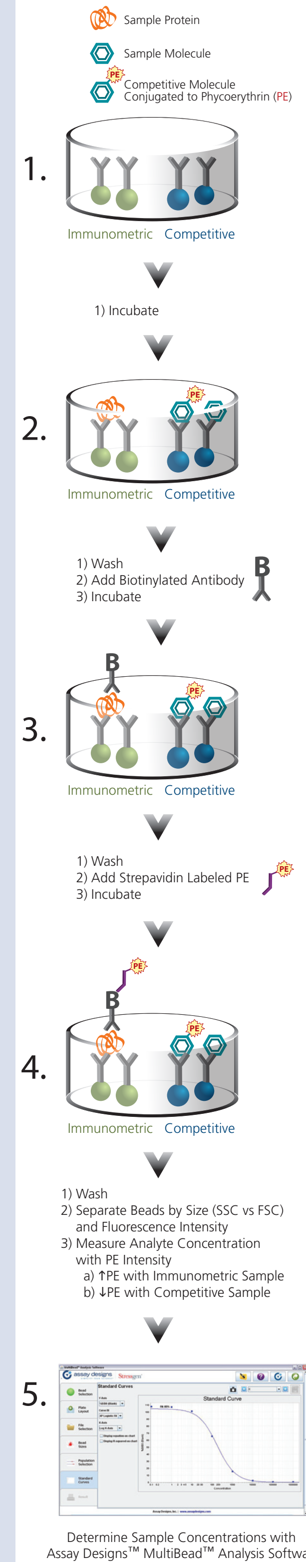
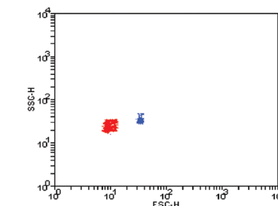
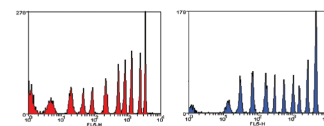


FIGURE 1

Measurement of 22 Analytes from One Sample. Two sets of beads with two sizes (4.0 and 5.4 um) with each having 11 fluorescent intensities were mixed together and separated by size with a BD FACSCalibur.



Each size population of beads is then separated by relative fluorescent intensity of each bead in the FL-5 channel.



The amount of 22 analytes could then be determined by the fluorescence associated with a phycoerythrin conjugate in the FL-2 channel.

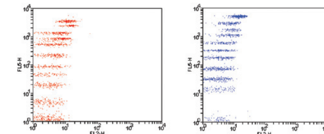
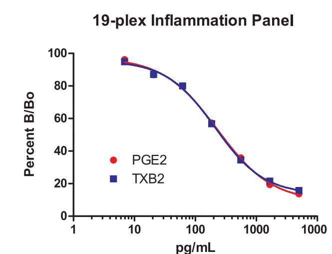
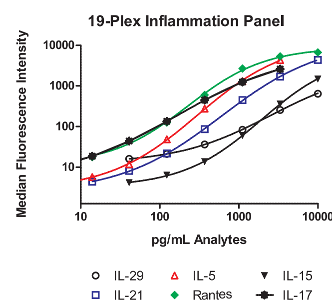


FIGURE 2

Titration curves for analytes included in the inflammation and heat shock multiplex immunoassay panels.





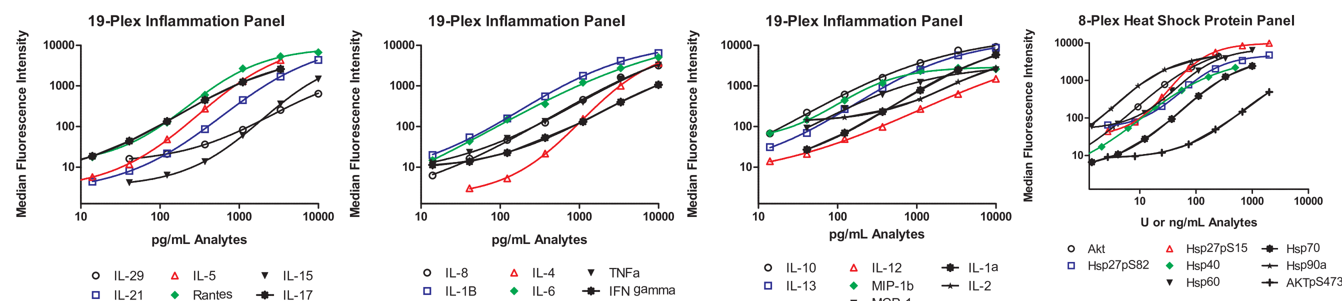


TABLE 1

	+IL-10	+IL-29	+IL-13	+IL-21	+IL-15	+IL-2	+IL-17a	+MCP-1	+IL-5	+IL-1a	+MIP-1B	+RANTES	+IL-12	+IL-8	+IL-4	+IL-6	+TNFa	+IFNg	+IL-1b
IL-10	5011	4	4	4	4	4	4	4	4	4	4	4	3	16	8	8	10	12	10
IL-29	24	1972	25	24	24	24	24	25	25	24	24	25	21	42	28	22	24	34	37
IL-13	12	12	7401	12	12	12	12	12	12	12	12	13	12	23	20	17	21	22	23
IL-21	32	42	34	3088	34	36	34	40	40	36	37	39	45	36	21	16	41	34	27
IL-15	79	90	81	89	5245	85	77	84	90	85	78	85	72	62	90	66	NT	44	72
IL-2	91	109	96	102	95	5024	90	104	103	100	94	102	85	90	51	50	63	77	68
IL-17a	13	14	14	14	15	15	2977	14	14	15	15	15	13	22	14	10	15	18	18
MCP-1	4	3	4	4	3	4	4	4542	4	6	3	4	3	8	5	8	6	7	5
IL-5	101	129	109	105	97	113	96	105	8112	111	90	108	106	247	195	268	233	218	223
IL-1a	4	4	4	4	4	6	4	5	4	7651	4	5	4	8	5	6	5	6	6
MIP-1B	156	162	160	154	146	148	146	163	157	150	3196	151	128	150	NT	121	108	104	129
RANTES	86	93	89	91	90	86	85	92	90	87	90	8202	71	64	51	57	63	51	55
IL-12	13	14	13	13	13	13	13	14	13	13	12	13	1193	19	13	15	15	16	15
IL-8	10	11	9	53	9	7	24	7	7	8	11	11	7	7018	9	6	10	11	14
IL-4	19	19	15	109	17	10	41	7	9	8	NT	14	9	27	7818	10	23	21	41
IL-6	294	274	305	467	261	215	292	10	12	13	17	17	11	379	264	3652	240	311	325
TNFa	18	18	15	112	NT	10	33	8	9	9	13	13	8	22	13	10	9405	18	30
IFNg	164	146	152	244	178	105	120	114	99	115	78	87	72	225	120	109	182	2175	217
IL-1b	12	12	9	68	10	11	10	7	7	7	10	9	7	8	5	7	6	6	6895

Single analyte drop in validation. Each antigen represented in the inflammation multiplex panel was spiked individually into the assay at a concentration of 10ng/mL. The spiked analytes are indicated horizontally across the top of the table. The readout assay is indicated down the left side of the table. The median fluorescence intensity for each analyte under each individual spike in is provided. Increase in background fluorescence for analytes other than the spiked analytes indicates potential cross-reactivity. The multiplex assays are further validated (data not shown) using single biotinylated antibody drop in experiments (all analytes present), single Ag drop out experiments (all analytes except one present) and single conjugate drop out experiments (all analytes present). These experiments validate the platform for additive cross reactivity attributed to both antigens and detector antibodies.

TABLE 2: IL-6

Dilution Factor	Assay Buffer	Serum	Plasma	Urine	Medium
Neat	81	70	81	68	73
1:2	75	77	81	94	88
1:4	78	84	81	87	83
1:8	88	88	97	112	88
1:16	100	100	100	123	87

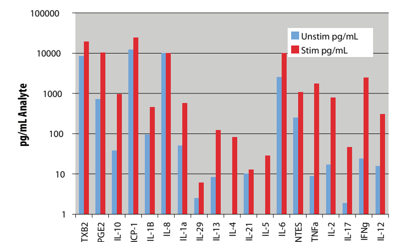
Dilutional linearity matrix validation. To validate performance in biological matrices, analytes were spiked into each matrix at 10ng/mL, serially diluted and run in the assay. The percent recovery in each matrix at each dilution is provided. Significant over or under recovery would indicate matrix interference. Representative dilutional linearity data for the IL-6 assay are provided as an example.

TABLE 3: IL-6

Sample Matrix (# of samples)	Minimum Required Dilution	Spike Concentration (pg/mL)	Recovery of Spike (Range)
Medium (n=1)	1:2	3333 370 50	90% 99% 90%
Plasma (n=5)	1:2	3333 370 50	114% 98% 105%
Serum (n=5)	1:2	3333 370 50	76% 109% 110%

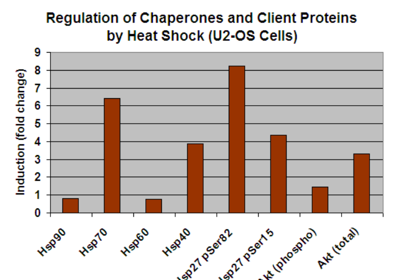
Spike and recovery matrix validation. Matrices were spiked at low, medium and high concentrations of analyte. Analyte recoveries were reported after subtracting out any endogenous analyte. Significant over or under recovery would indicate matrix interference. Representative spike and recovery data for the IL-6 assay are provided as an example.

FIGURE 3



Mitogen stimulation of peripheral blood mononuclear cells (PBMCs) for production of inflammation markers. PBMCs were cultured for 48 hours in the presence and absence of 50 µg/mL PHA and Pokeweed extract. The culture supernatants were sampled and diluted to within range of the individual assays included in the inflammation multiplex panel.

FIGURE 4



Stress response of a human osteosarcoma cell line characterized by MultiBead analysis. U2-OS cells were subjected to heat-shock at 42 °C for 2 hours, followed by an 18 hour recovery at 37 °C. The cells were lysed in a buffer containing 25 mM TrisHCl, pH 7.6, 150 mM NaCl, 1% NP-40, 1% deoxycholate, and 0.1% SDS. Samples containing approximately 1 mg/ml total cellular protein were diluted 4- to 16-fold into assay buffer for Multibead analysis. Changes in the levels of molecular chaperones and client proteins are expressed as fold induction relative to a non-heat shocked control sample.

CONCLUSIONS

1. The MultiBead immunoassay platform enables multiplex detection of both extracellular and intracellular targets.
2. MultiBead immunoassays enable simultaneous detection of small molecules and proteins by combining competitive and sandwich immunoassay formats.
3. MultiBead immunoassays have been validated in a variety of matrices.
4. The multiplex assay format provides specificity and biologically relevant sensitivity.
5. The MultiBead data analysis software is provided free and offers a customizable, flexible and easy to use user interface.