Bio-Plex® suspension array system

tech note 5649

Development and Validation of a Novel Multiplex Immunoglobulin Isotyping Assay on Magnetic Microspheres

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Introduction

Monitoring immunoglobulin (Ig) concentrations is a valuable approach in the evaluation of immune responses. Blood Ig concentrations vary depending on factors such as inheritance, disease state, drug treatment, and Ig class and subclass. Ig isotypes can be differentiated into classes and subclasses based on the domains of heavy and light chains in the constant region of the antibody molecule. Each Ig isotype plays a specific role in the immune system by binding to receptors on various cell types and mediating events such as complement activation and pathogen clearance (Hamilton 2001).

We developed a multiplex sandwich immunoassay based on microspheres (beads), that simultaneously quantitates the concentrations of seven Ig isotypes: IgG_1, IgG_2, IgG_3, IgG_4, IgA, IgE, and IgM (Figure 1). This Bio-Plex Pro™ human isotyping 7-plex panel determines concentrations of these seven isotypes from a single sample of serum, plasma, or tissue culture supernatant, with a recommended dilution of 1:10,000 in isotyping diluent. The multiplex format yields Ig profiles of the isotypes with significant time and cost savings over technologies such as ELISA, radial immunodiffusion, and nephelometry. A complete profile of Ig isotype concentrations from as little as 10 µl of sample is obtained in about 3 hours.

In this report, we describe results of validation experiments, calculating the sensitivity and upper and lower limits of quantitation for the assay.

We validated the isotyping panel using samples from healthy humans and patients with rheumatoid arthritis (RA). We tested the assay against World Health Organization (WHO) reference standards: National Institute for Biological Standards and Control (NIBSC) 67/086 for IgG, IgA, and IgM and NIBSC 75/702 for IgE, and compared isotype concentrations across experiments and methods.

Methods

We studied the performance characteristics of the Bio-Plex Pro human isotyping panel and compared isotype concentrations across experiments and methods. Standards containing known concentrations of purified human Igs were prepared by adding 0.5 ml isotyping diluent to lyophilized standard (both provided in the Bio-Plex Pro kit) and incubating on ice for 30 min. Starting concentrations were: IgG1, 12,000 ng/ml; IgG2, 6,000 ng/m; IgG3, 5,000 ng/ml; IgG4, 1,000 ng/ml; IgA, 2,000 ng/ml; IgE, 100 ng/ml; and IgM, 1,500 ng/ml. Isotyping diluent was used to make 1:4 serial dilutions for a total of eight standard concentrations.
Per plate, 250 µl of antibody-conjugated beads, 312.5 µl of detection antibody, and 62.5 µl of streptavidin-PE were used.

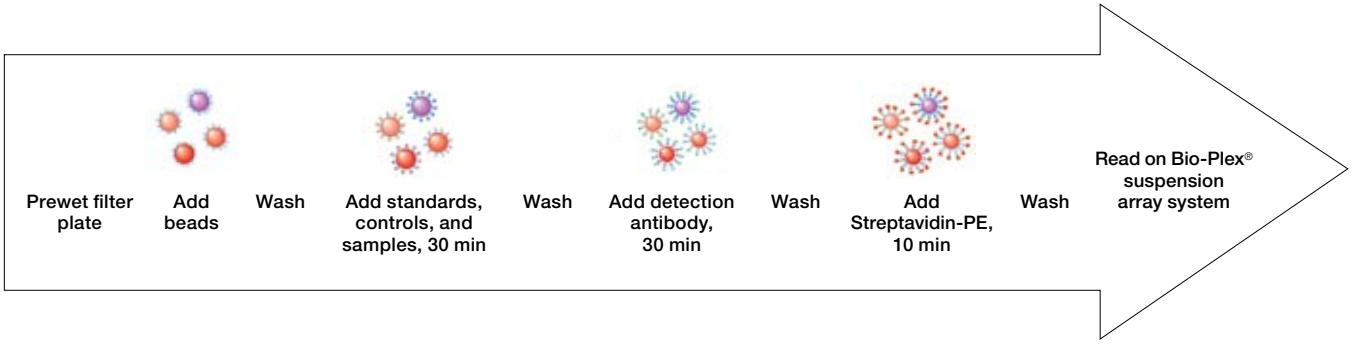

Fig. 1. Schematic representation of the experimental workflow for a multiplex bead-based sandwich immunoassay.

Controls were prepared by adding 1 ml isotyping diluent to lyophilized control and incubating on ice for 30 min. The controls were spiked at three different concentrations (high, medium, and low) and a 5 µl serum sample from a healthy subject (diluted 1:10,000 in isotyping diluent) were run using 250 µl antibody-conjugated beads, 312.5 µl detection antibody, and 62.5 µl streptavidin-PE.

Igs were spiked into single donor serum and plasma to determine recovery in the sample matrix. Once these spiked samples were diluted 1:10,000, the concentration of each spike was: IgG1, 700 ng/ml; IgG2, 350 ng/ml; IgG3, 300 ng/ml; IgG4, 60 ng/ml; IgA, 100 ng/ml; IgE, 5; and IgM, 90 ng/ml. In addition, World Health Organization (WHO) reference standards (pooled sera), were run using 250 µl antibody-conjugated beads, 312.5 µl detection antibody, and 62.5 µl streptavidin-PE per plate.

We tested the assay against National Institute for Biological Standards and Control (NIBSC) 75/702 for IgE and NIBSC 67/806 for IgG, IgA, and IgM. Target activities were based on the reconstitution volumes of the WHO reference samples, international units (IU) of activity in each vial as described by the product sheet, and the concentration results obtained from the Bio-Plex Pro human isotyping panel: IgG, 0.103 ng/IU; IgA, 0.019 ng/IU; IgE, 3 ng/IU; and IgM, 0.006 ng/IU.

Experiments were performed according to the Bio-Plex Pro isotyping reagent kit instructions (Figure 1). Runs were done in triplicate on a 96-well plate, and five independent verification assays were performed. Although the standard vacuum assay separation method was used, these magnetic bead-based assays also allow magnetic separation and automation of wash steps. This innovation eliminates the need for a vacuum manifold, greatly simplifying assay processing. Assays were read on the Bio-Plex suspension array system.

Results

Performance Characteristics of the Bio-Plex Pro Human Isotyping Panel

Sensitivity (limit of detection, LOD) for each target was calculated as the concentration of each standard corresponding to the background median fluorescence intensity (MFI) values plus two standard deviations. Lower limit of quantitation (LLOQ) and upper limit of quantitation (ULOQ) were defined for each target by determining the standard concentration range that met the assay's technical specifications for both precision (≤15% and ≤20% for intra- and inter-assay precision, respectively, as measured by percent coefficient of variation, %CV) and accuracy (70–130% of expected concentration). These results are shown in Table 1.

Table 1. Performance of the Bio-Plex Pro human isotyping 7-plex panel.*

Target	LOD, ng/ml	LLOQ, ng/ml	ULOQ, ng/ml
IgG_1	0.52	2.93	7,950
IgG_2	1.65	19.92	6,000
IgG_3	0.29	5.25	5,000
IgG_4	0.05	0.06	813
IgM	0.05	0.42	1,500
IgA	0.12	0.12	500
IgE	0.003	0.01	100

* Concentrations are for 10,000-fold diluted samples.

Intra-assay %CV was calculated from the MFI variance of three replicate wells on a 96-well plate for each of eight standard concentrations. The mean intra-assay %CV for the five verification assays is shown in Table 2. The technical specifications (≤15%) were achieved for all points.

Inter-assay %CV was determined from the variance of measured concentration values of three Ig-spiked controls (high, medium, and low) and a healthy serum sample, run on five verification plates. The Ig spiked controls are a mixture of the 7 Igs spiked into isotyping diluent. Data are shown in Table 3. The technical specifications (≤20%) were met as required for the medium- and low-spike concentrations, and for all but two targets for the high-spike concentration.

Accuracy of the assay was determined from the mean percent recovery of Ig-spiked controls and Igs spiked into both single-donor serum and single-donor plasma, in triplicate, over the course of five verification runs. These results are shown in Table 4 and Table 5. The WHO reference standards results are shown in Table 5.

Table 2. Intra-Assay Precision. The mean intra-assay % CV was taken for each standard curve point and a serum sample.

IgG1		IgG2		IgG3		IgG4		IgM		IgA		IgE	
Std Conc. (ng/ml)	% CV	Std Conc. (ng/ml)	% CV	Std Conc. (ng/ml)	% CV	Std Conc. (ng/ml)	% CV	Std Conc. (ng/ml)	% CV	Std Conc. (ng/ml)	% CV	Std Conc. (ng/ml)	% CV
12,000	6.6	6,000	8.3	5,000	6.1	1,000	7.9	1,500	6.1	2,000	3.5	100	5.4
3,000	2.1	1,500	2.8	1,250	2.3	250	2.2	375	3.2	500	1.7	25	2.2
750	2.7	375	4.7	312.5	3.3	62.5	2.9	93.75	7.3	125	2.5	6.25	4.1
187.5	3.2	93.75	5.4	78.13	4.8	15.63	3.6	23.44	6.7	31.25	2.8	1.56	2.9
46.88	4.4	23.44	5.2	19.53	4.4	3.91	3.4	5.86	8.6	7.81	3.3	0.39	3.7
11.72	5.4	5.86	6.8	4.88	6.4	0.98	5.1	1.46	8.5	1.95	7.5	0.1	6.2
2.93	3.9	1.46	6.3	1.22	5.8	0.24	4.9	0.37	7.9	0.49	4.4	0.02	6.3
0.73	4.0	0.37	3.8	0.31	7.4	0.06	6.3	0.09	4.9	0.12	5.1	0.01	7.1
Serum	1.99	Serum	5.15	Serum	2.83	Serum	1.85	Serum	6.51	Serum	1.26	Serum	3.44

Table 3. Inter-Assay Precision and Recovery. High, medium, and low spiked control concentrations and a serum sample's concentration are listed for each target.

Target	Sample and Expected Concentration (ng/ml)	Mean	SD	Mean Inter-assay % CV	% Recovery
IgG1	4,800	4,113	572	13.9	86
	600	524	41	7.8	87
	40	38	2	5.8	94
	Serum	7,015,434	997,624	14.2	
IgG2	2,400	1,681	289	17.2	70
	300	251	20	8.0	84
	20	18	3	17.4	90
	Serum	3,440,978	624,721	18.2	
IgG3	2,000	2,015	518	25.7	101
	250	223	14	6.4	89
	16.67	16.20	2	9.5	97
	Serum	457,902	65,508	14.3	
IgG4	400	332	22	6.5	83
	50	46	3	6.2	92
	3.33	3.17	0	7.3	95
	Serum	430,152	60,426	14.0	
IgM	600	485	49	10.0	81
	75	66	6	9.0	88
	5	4	0	8.5	89
	Serum	684,591	84,751	12.4	
IgA	800	607	135	22.2	76
	100	93	10	10.2	93
	6.67	6.44	0	6.1	97
	Serum	1,168,078	153,261	13.1	
IgE	40	37	2	5.2	92
	5	4.55	0	7.0	91
	0.33	0.33	0	7.7	99
	Serum	1,111	173	15.6	

Table 4. Accuracy of Ig quantitation in serum and plasma samples.*

Target	Spiked-In Concentration x 10^{-4}, ng/ml	% Recovery Serum	% Recovery Plasma
IgG_1	700	121	97
IgG_2	350	120	109
IgG_3	300	108	108
IgG_4	60	87	72
IgM	90	110	104
IgA	100	143	100
IgE	5	99	100

* Accuracy for spiked Igs was determined by using the formula, [(observed concentration of sample + Ig spike) – (observed concentration of sample)]/(observed concentration of Ig spike) x 100. Mean values from five verification runs are shown.

Table 5 . Quantitation of IgG, IgA, IgM, and IgE in WHO reference standards.*

Target	Activity (ng/IU)	WHO Standard Concentration, mg/ml NIBSC 67/086 (IgG, IgA, IgM)	WHO Standard Concentration, mg/ml NIBSC 75/702 (IgE)
IgG_1	0.103	10.69	8.62
IgG_2	0.103	7.84	5.04
IgG_3	0.103	1.61	5.60
IgG_4	0.103	0.65	0.68
IgA	0.019	3.82	4.29
IgE	3.00	0.00067	0.03061
IgM	0.006	1.20	0.65

* Based on the reconstitution volumes of the WHO reference samples, international units (IU) of activity in each vial as described by the product sheet, and the concentration results obtained from the Bio-Plex Pro human isotyping panel, IgE activity was 3.210 ng/IU, IgG was 0.103 ng/IU, IgA was 0.019 ng/IU, and IgM was 0.006 ng/IU. WHO reference standards NIBSC 67/086 and 75/702 were used.

Validation of Healthy and Diseased Sera and Plasma

We obtained 60 serum, 60 EDTA plasma, and 10 sodium citrate plasma samples from 130 different, classified healthy patients and analyzed them using the Bio-Plex Pro human isotyping panel. Histograms with n < 130 contained out-of-range values (i.e. were undetectable) for some targets. Measured concentrations of target were similar for serum and plasma samples, so data combined from both serum and plasma are shown in Figure 2.

We compared Ig profiles from subjects that were healthy or had RA. We analyzed 20 lots of sera and plasma from individuals that were classified as healthy (16 males and 4 females, ages 21–48) and 20 lots from individuals diagnosed with RA (20 males, ages 24–70), totaling 40 different patient samples. Increased values were observed for all IgG subclasses, IgM, and IgA in RA samples relative to healthy samples (Figure 3). These results correlate with trends cited in the literature (Adhya et al. 1998). It is known that the distribution of antibodies specific for rheumatoid factors is predominantly IgG1 and IgG4 (Chapuy-Regaud et al. 2005), and this may explain the observed higher concentrations for these IgG subclasses when compared to healthy controls. Interestingly, IgG2 and IgA levels were also higher in RA patients versus controls. It is not known whether any of the RA patients were undergoing treatment that could have influenced their Ig profiles.

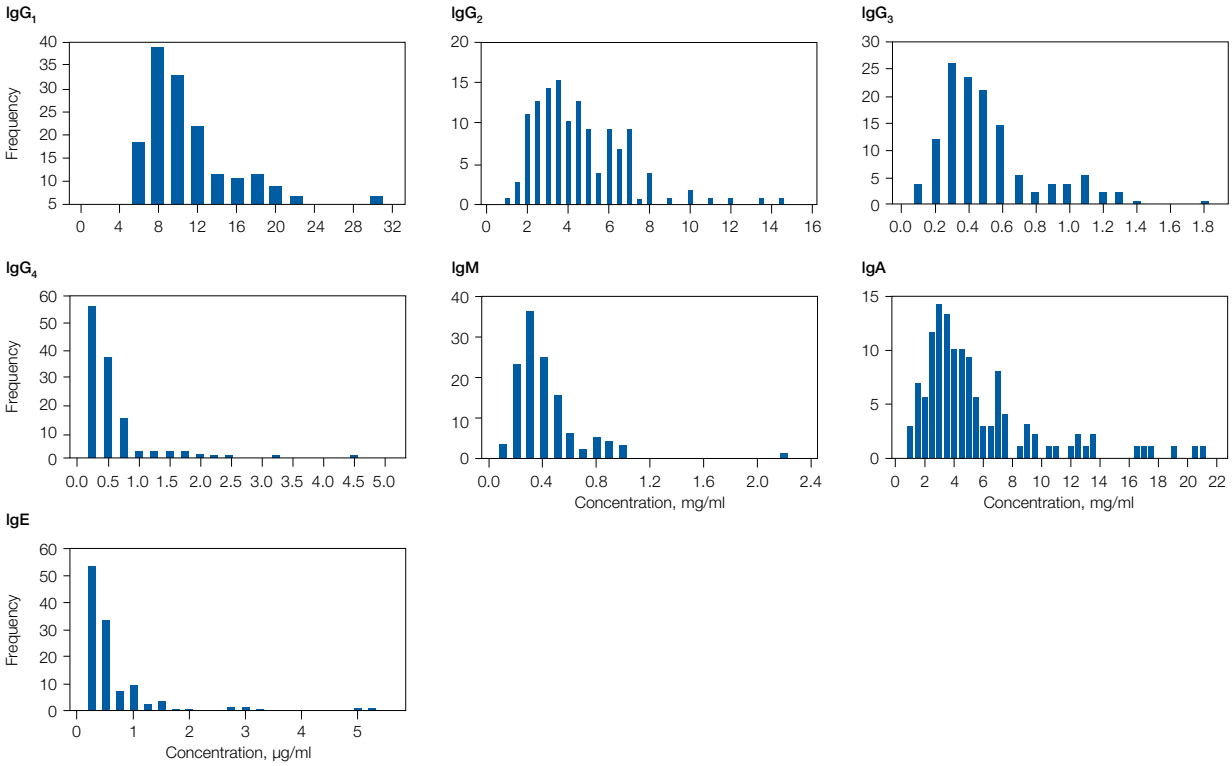

Fig. 2. Concentrations of seven Ig isotypes in serum and plasma samples of 130 healthy humans. Owing to occasional out-of-range values, n was 126 for IgG_4, 128 for IgA, and 127 for IgE.

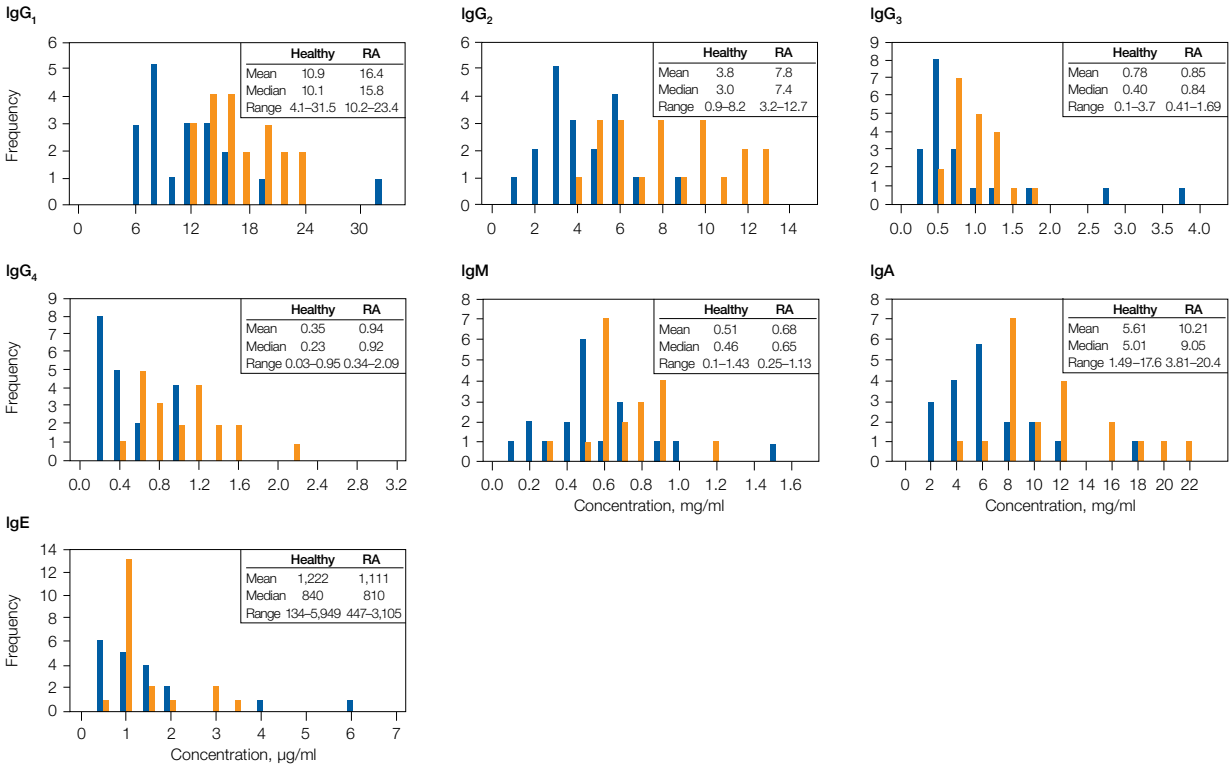

Fig. 3. Comparison of Ig concentrations in serum and plasma samples between healthy human subjects (■, n = 20) and subjects with RA (■, n = 20). Histograms show distribution of isotypes in healthy vs. arthritic samples. Inset tables show mean, median, and range of Ig concentrations in healthy and RA patients.

Conclusions

The Bio-Plex Pro human isotyping 7-plex panel was used to determine the isotype profiles of seven Ig classes and subclasses in a single, small volume of sample. The assay panel proved specific (0–3% cross-reactivity), precise (≤15% intra-assay and ≤20% inter-assay CV within working assay range), and accurate (70–130% recovery) for samples at a 1:10,000 dilution. It is also linear for most targets at sample dilutions of 1:5,000 and 1:2,500 (data not shown). The suggested 1:10,000 dilution resulted in a robust assay. Diluting the sample matrix to this degree resulted in negligible matrix effects, making the assay suitable for diverse applications. Assay ranges allowed the measurement of normal and diseased samples. This enables evaluation of isotype profiles of different populations and the monitoring of effects of drug treatments.

The panel can be used with traditional vacuum filtration washing or can be adapted to a nonvacuum, magnetized, automated system for higher throughput. The flexibility and ease of use of the Bio-Plex Pro human isotyping 7-plex panel makes it ideal for a variety of different lab settings.

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Information in this tech note was current as of the date of writing (2007) and not necessarily the date this version (rev A, 2008) was published.

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Bulletin 5649 Rev A US/EG *07-0805 0508 Sig 0308*