Bio-Plex® suspension array system

tech note 6394

Development and Validation of a Newly Reconfigured Bio-Plex Pro™ Human Isotyping Assay Panel

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Introduction

Immunoglobulins, commonly known as antibodies, are produced by human plasma cells for humoral immunity, targeting foreign material or antigens such as bacteria and viruses (August 2006). Immunoglobulins are classified into five isotypes: IgM, IgA, IgE, IgD, and IgG (Figure 1). IgG is further divided into four subclasses (IgG_1, IgG_2, IgG_3, and IgG_4) in humans, named in order of their abundance in serum. Complete or near complete loss of certain species of immunoglobulin in some primary immunodeficiency syndromes was found to increase the risk of certain types of disorders and infections. Hence profiling of immunoglobulin isotypes can greatly benefit researchers studying allergy, cancer, autoimmune and infectious diseases, and vaccine and drug development (Furst 2008).

A premixed panel of multiplexed human isotyping assays was developed to simultaneously quantify two isotypes (IgA and IgM) and four IgG subclasses (IgG_1, IgG_2, IgG_3, and IgG_4) in human serum, plasma, and cell culture supernatant. IgE and IgG total assays were configured in a singleplex format due to IgE's different sample dilution factor and IgG total's cross-reactivity to IgG subclasses in the premixed 6-plex panel. The assays were designed on the Luminex MagPlex 6.5 µm beads. Utilizing xMAP magnetic bead technology, the Bio-Plex® systems (Bio-Plex 200, Bio-Plex® MAGPIX™, and Bio-Plex 3D) can measure the level of seven human immunoglobulin isotypes in less than four hours using as little as 5 µl of serum or plasma samples. The performance of these eight isotyping assays was evaluated according to standard verification and validation parameters, including assay sensitivity, precision, accuracy, working ranges, and specificity and linearity of dilutions. The assays were further tested on various types of disease samples such as asthma, cancers, pneumonia, and sepsis along with normal samples.

Method

The isotyping assays are configured into a convenient all-in-one kit format that includes assay and reagents. The recommended human serum and plasma sample dilution factor is 1:40,000 for the 6-plex as well as IgA, IgM, and IgG total singleplex assays. IgE is present at the lowest serum concentration and its recommended dilution factor is 1:500. All the dilutions are carried out using the provided isotyping diluent. The assays employ a standard sandwich enzyme immunoassay method using a 96-well plate format.

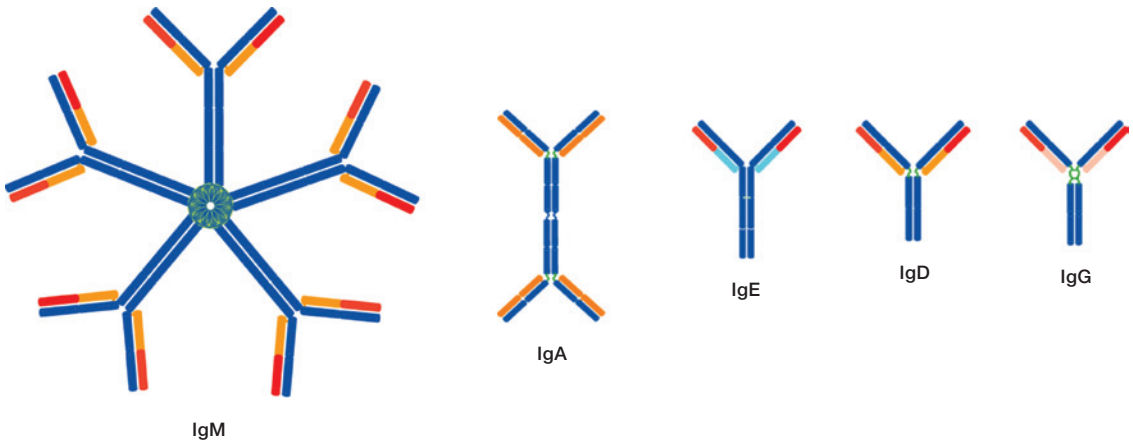

Fig. 1. Different isotypes of immunoglobulins.

The capture antibody-coupled beads are allowed to react with a sample containing target immunoglobulins of interest. After performing a series of washes to remove unbound material, a biotinylated detection antibody specific for a different epitope on the target is added to the beads. The result is the formation of a sandwich of antibodies around the specific target. The reaction mixture is detected by the addition of a reporter dye, streptavidin-phycoerythrin (SA-PE), which binds to the sandwich complexes via the biotinylated detection antibodies. The contents of each well are drawn up into the Bio-Plex suspension array reader, which identifies and quantifies each specific reaction based on bead color and fluorescence signal intensity (Figure 2). All washes were performed using a Bio-Plex Pro™ wash station. Data acquisition was performed using the Bio-Plex 200 system at low PMT setting, the Bio-Plex 3D system at standard PMT setting, or the Bio-Plex MAGPIX system using the default setting.

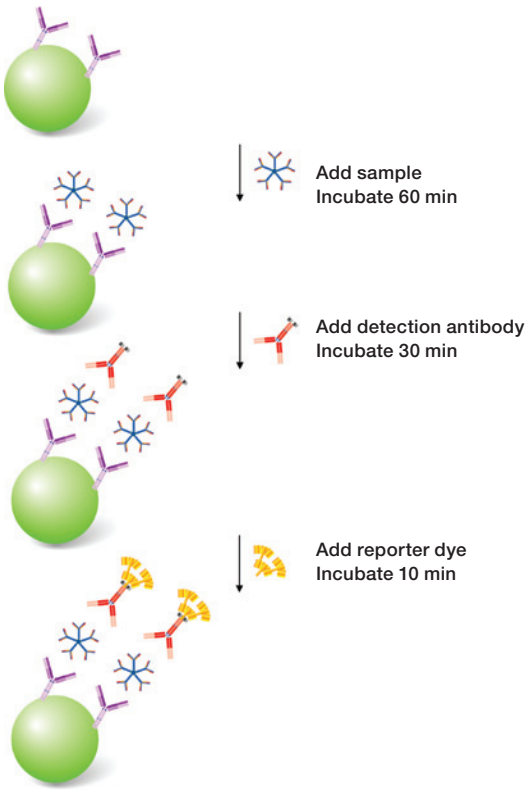

Fig. 2. Schematic representation of an isotyping assay workflow.

Performance characteristics of the isotyping assays were examined according to the following parameters: assay specificity, sensitivity, precision profile, accuracy, assay working range, matrix effects (linearity of dilution and parallelism), and validation with biological samples.

Assay specificity was examined by performing single-antigen and single-detection cross-reactivity weighted on the second highest standard point. Single-antigen study evaluates the specificity of a capture antibody. This was conducted by testing an individual antigen in the presence of multiplexed capture beads and detection antibodies. Single-detection study evaluates the specificity of the detection antibody. This was conducted by testing the individual detection antibody in the presence of multiplexed antigens and capture beads. With the exception of the IgG_1 capture antibody, which showed a mild cross-reactivity to the IgG_2 antigen, no cross-reactivity was detected in the rest of the assays (Table 1).

Table 1. Single-antigen and single-detection cross-reactivity.

Single Antigen	% of Cross-Reactivity						
	IgG_1	IgG_2	IgG_3	IgG_4	IgA	IgM	IgE
IgG_1	—	0.0	0.0	0.1	0.1	0.0	0.1
IgG_2	2.8	—	0.3	0.1	0.2	0.0	0.0
IgG_3	0.0	0.0	—	0.3	0.3	0.0	0.0
IgG_4	0.1	0.0	0.0	—	0.1	0.0	0.1
IgA	0.0	0.0	0.0	0.0	—	0.1	0.0
IgM	0.1	0.1	0.0	0.1	0.2	—	0.2
IgE	0.0	0.0	0.0	0.0	0.0	0.0	—
Single Detection	**% of Cross-Reactivity**						
	IgG_1	IgG_2	IgG_3	IgG_4	IgA	IgM	IgE
IgG_1–G_4	—	—	—	—	0.4	0.0	0.1
IgA	0.0	0.0	0.0	0.0	—	0.0	0.0
IgM	0.0	0.0	0.0	0.0	0.2	—	0.1
IgE	0.0	0.0	0.0	0.0	0.0	0.0	—

— Not applicable

Assay sensitivity, defined as limit of detection (LOD), was evaluated by adding two standard deviations to the median fluorescence intensity (MFI) of a background. The mean of three independent assays was calculated for isotyping diluent and only one assay was run on RPMI. The assays showed comparable performance, with the exception of IgG_2, in RPMI cell culture media (Table 2).

Reproducibility was reported in both intra- and inter-assay precision in isotyping diluent and/or RPMI matrices (Table 3). Intra-assay precision was calculated as the coefficient of variation (%CV) on three replicate wells of standard curve points on a single assay plate. Inter-assay precision was calculated as the %CV of the spiked controls from three independent assays.

Assay accuracy (also defined as recovery) was calculated as the percentage of the observed concentration value of a spiked standard of known concentration relative to the expected value. This parameter was evaluated using standard points and spiked controls in both multiplex and singleplex configurations using isotyping diluent. Overall, the standard recovery is comparable in both configurations, with most targets recovering within 80–120% on at least seven of the eight standard points (Table 4).

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Table 2. Limit of detection (LOD).

Analyte		IgG_1	IgG_2	IgG_3	IgG_4	IgA	IgM	IgE	IgG Total
LOD (ng/ml)	Isotyping Diluent	0.0264	0.6615	0.0030	0.0021	0.1034	0.1473	0.0040	2.1603
	RPMI	0.0076	28.02	0.0095	0.0094	0.0799	0.1358	0.0061	3.0675

Table 3. Precision profile for intra- and inter-assay %CV.

	Intra-Assay Precision (%CV)			Inter-Assay Precision (%CV)	
	Isotyping Diluent Matrix		RPMI Matrix	Isotyping Diluent Matrix	
Analyte	Multiplex	Singleplex	Multiplex	Standard Points	Spike Controls
IgG_1	2	▲	6	1	4
IgG_2	4	▲	9	3	5
IgG_3	3	▲	3	2	5
IgG_4	4	▲	3	4	5
IgA	5	5	5	3	3
IgM	3	2	4	2	4
IgE	●	3	4	3	14
IgG Total	●	4	7	5	3

● Multiplex not available

▲ Singleplex not available

Table 4. Assay accuracy (standard and spiked recovery).

	Recovery (%) of Isotyping 6-plex in Isotyping Diluent						Singleplex	
Analyte	IgG_1	IgG_2	IgG_3	IgG_4	IgA	IgM	IgE	IgG Total
Standard 1	91	—	84	83	—	94	82	—
Standard 2	108	113	116	116	87	103	118	81
Standard 3	99	95	99	96	93	99	96	100
Standard 4	98	102	97	100	103	101	99	103
Standard 5	102	100	102	103	101	98	103	99
Standard 6	100	100	101	98	99	103	99	99
Standard 7	98	101	99	100	101	98	100	104
Standard 8	101	99	100	100	99	101	100	95
Spike 1	97	85	107	100	96	99	86	109
Spike 2	97	85	104	104	92	95	86	106
Spike 3	101	97	103	97	101	98	93	95
Spike 4	100	92	111	107	102	100	95	94
Spike 5	98	88	103	102	88	95	97	85
Spike 6	98	88	99	96	85	89	91	103
Spike 7	90	86	97	—	84	94	89	86

— Not within 80–120% recovery range

Assay working range is defined as the range between the lower limit of quantification (LLOQ) and the upper limit of quantification (ULOQ) in which an assay is both precise (intra-assay %CV ≤10% and inter-assay %CV ≤15%) and accurate (80–120% recovery). The results, including dynamic range, are summarized in Table 5.

Linearity of dilution ensures that analytes present in concentrations above the ULOQ can be diluted and measured accurately within the assay working ranges. Due to high endogenous levels, prior to a threefold serial dilution, pooled normal human serum or plasma samples were pre-diluted 3,000-fold for the 6-plex as well as the IgA and IgM assays, 1,000-fold for the IgG total assay, and 16-fold for the IgE assay. The linear relationship of observed concentration and expected concentration of each dilution

Table 5. Assay working range and dynamic range.

Analyte	IgG_1	IgG_2	IgG_3	IgG_4	IgA	IgM	IgE	IgG Total
ULOQ (ng/ml)	2,107	11,227	863	200	532	6,376	406	30,270
LLOQ (ng/ml)	0.13	2.74	0.07	0.02	0.13	0.78	0.02	3.00
Dynamic range (log)	4	3	4	4	3	3	4	4

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Table 6. Assay linearity and parallelism.

	Linearity R^2 Values				Parallelism					
					Slope			Slope		
	Serum		Plasma							
Analyte	Multiplex	Singleplex	Multiplex	Singleplex	Isotyping Diluent	Diluted Serum	% Difference in Slope	Isotyping Diluent	Diluted Plasma	% Difference in Slope
IgG_1	0.9996	▲	0.9996	▲	0.95	0.94	0	0.95	0.92	3
IgG_2	1.0000	▲	1.0000	▲	1.07	1.13	6	1.07	1.11	4
IgG_3	1.0000	▲	0.9976	▲	0.88	0.87	2	0.88	0.89	1
IgG_4	0.9917	▲	0.9904	▲	0.95	0.97	2	0.95	0.95	0
IgA	0.9993	0.9983	0.9995	0.9997	1.14	1.23	8	1.14	1.23	8
IgM	1.0000	1.0000	1.0000	1.0000	0.98	0.97	1	0.98	1.01	3
IgE	●	0.9999	●	0.9995	0.92	0.83	9	0.92	0.85	8
IgG Total	●	0.9994	●	0.9999	1.05	1.05	1	1.05	1.09	3

● Multiplex not available

▲ Singleplex not available

point within the assay working range was plotted with an R^2 value reflecting the linearity of sample dilution (Table 6).

Parallelism was investigated by comparing the slope of a sample curve (human serum/plasma diluted in isotyping diluent) with the slope of a standard curve prepared in isotyping diluent. The percentage of difference in the slope (slope of the tangent at midpoint) between the two curves is tabulated in Table 6.

Validation with Biological Samples

The isotyping 6-plex assay and the IgE and IgG total singleplex assays were further validated with serum and plasma samples from normal controls and various disease types including asthma, breast cancer, colon cancer, myeloma, pneumonia, sepsis, and B-cell chronic lymphatic leukemia.

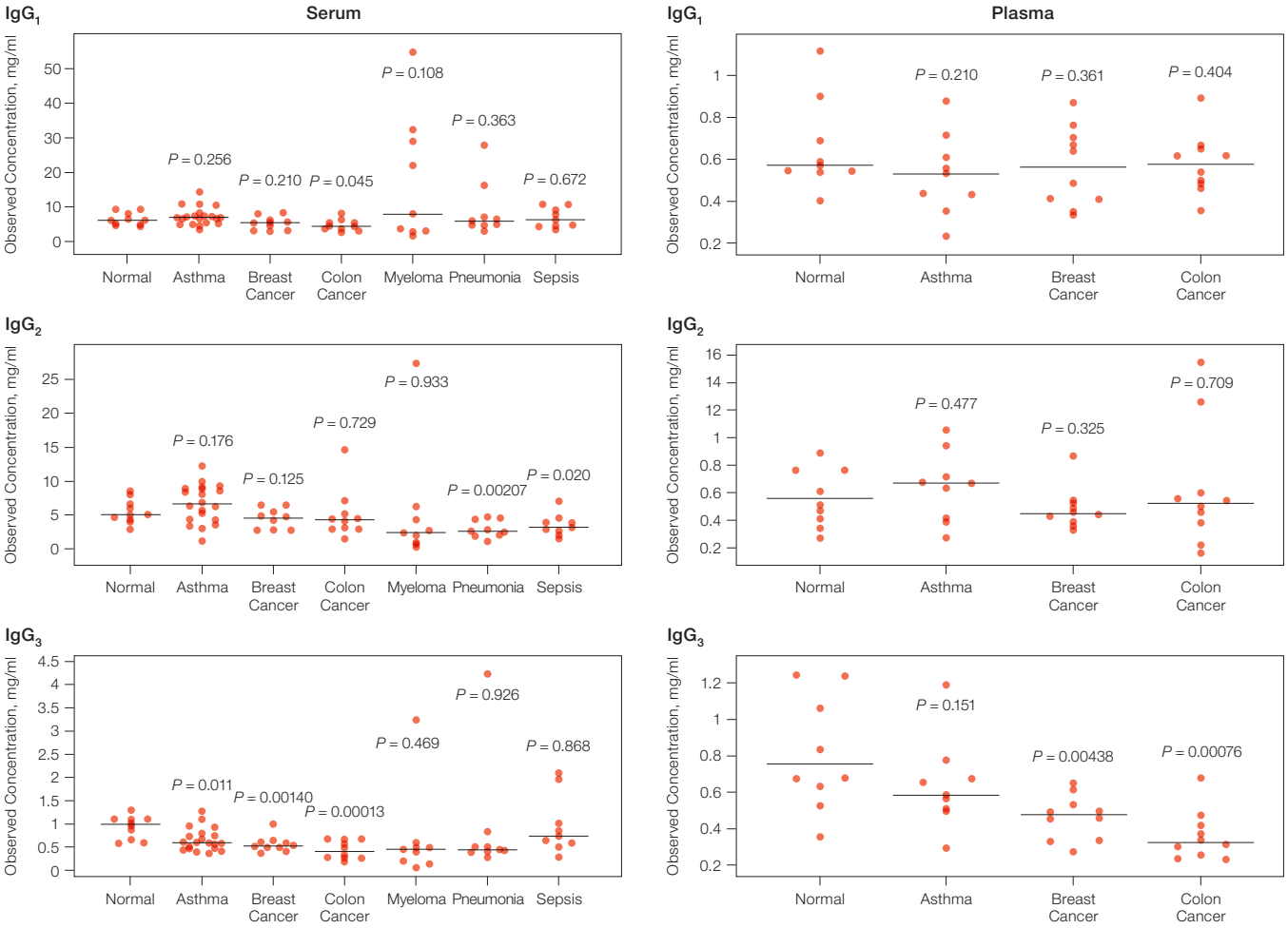

Fig. 3 continued on next page.

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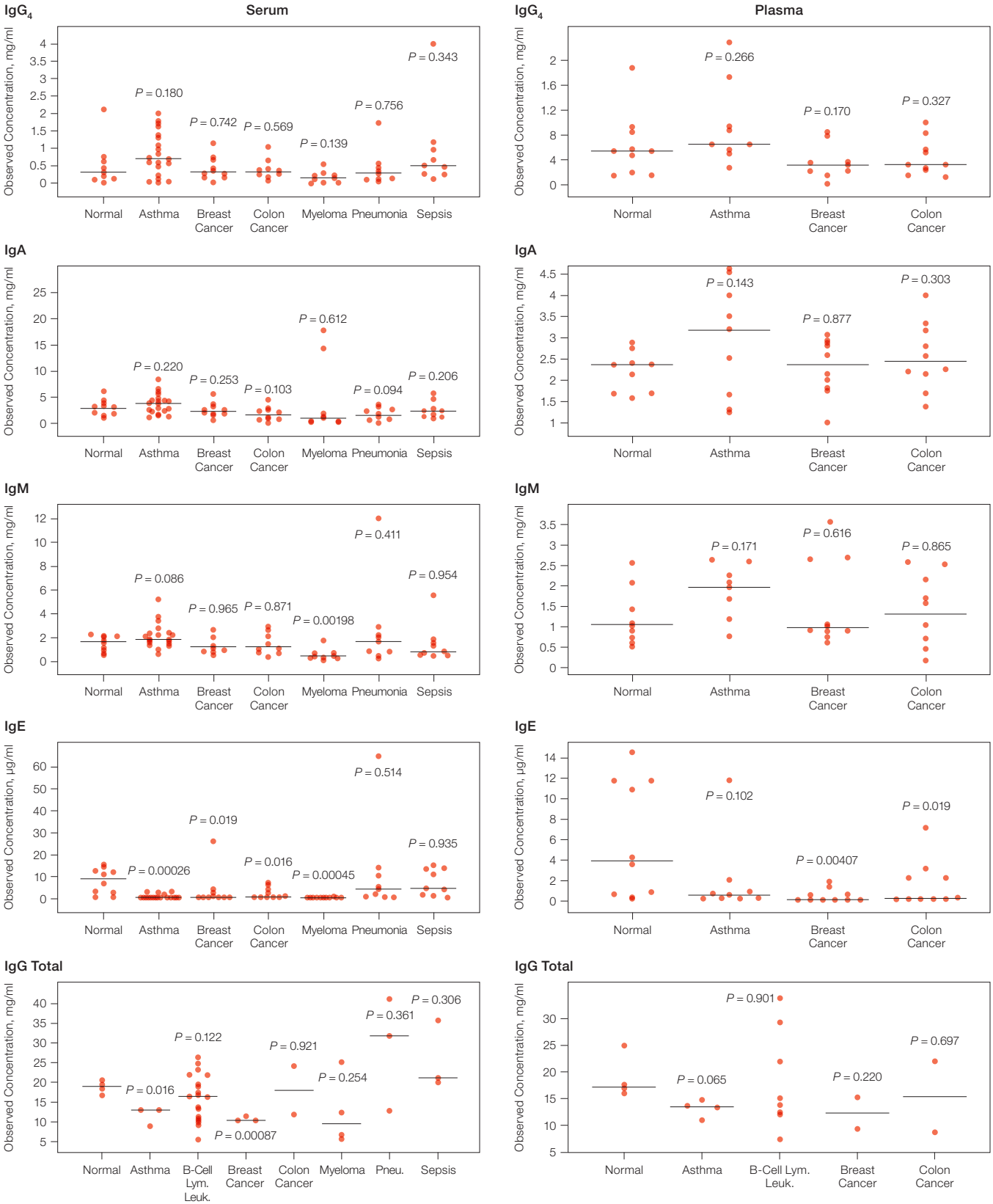

Fig. 3. Sample validation with serum and plasma collected from healthy subjects and individuals with various disease types. Non-matching normal serum and plasma samples were used as the baseline reference. Bio-Plex Data Pro™ software was used to calculate statistical differences by t-test, where a p-value <0.05 indicates statistical significance. Non-numeric sample values were not included in the calculation.

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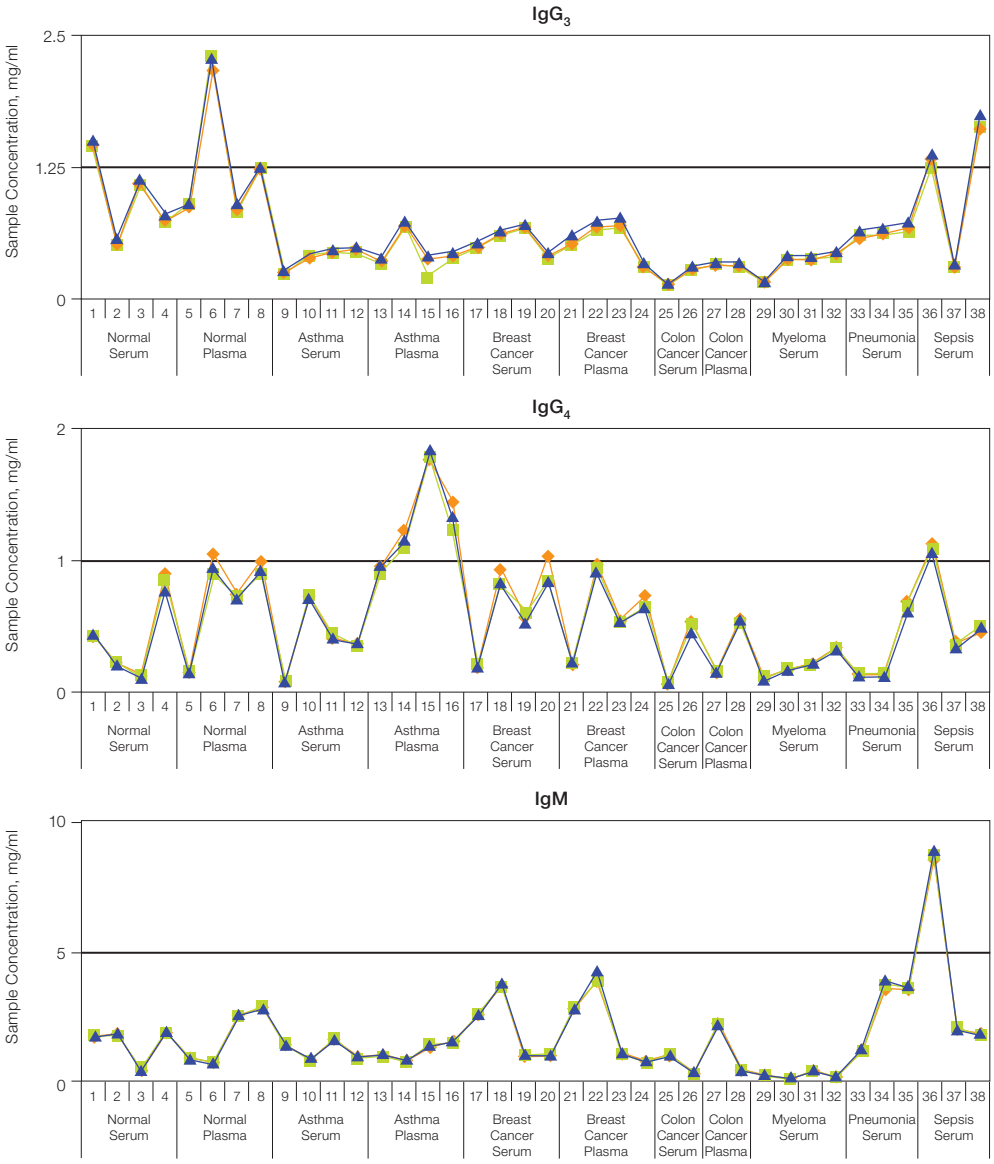

Fig. 4. Alignment in sample readout. 38 samples (healthy and diseased combined) were recorded by Bio-Plex 200 (◆), Bio-Plex 3D (■), and Bio-Plex MAGPIX (▲) system respectively.

Performance Alignment: Bio-Plex 200, Bio-Plex MAGPIX, and Bio-Plex 3D Instruments

To enable end users the flexibility of choosing other Bio-Plex systems, the isotyping assays were evaluated on the Bio-Plex 200, Bio-Plex MAGPIX, and Bio-Plex 3D systems. Excellent agreement in sample concentration was recorded on all three platforms (Figure 4).

Conclusions

We have developed a Luminex-based human isotyping panel suitable for simultaneous measurement of multiple isotypes and IgG subclasses in various sample matrices. The assays have shown excellent specificity, precision, accuracy, sensitivity, and have achieved technical specifications. The data from present experiments are consistent with published reports that levels of related isotypes often decrease under disease conditions.

References

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