Bio-Plex® suspension array system

tech note 6119

Multiplex Analysis of Rat Cytokines and Diabetes Biomarkers Using Bio-Plex Pro™ Rat Cytokine and Diabetes Assays

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Introduction

The rat is second only to the mouse as an animal model for immunological research. Like mice, rats lend themselves to a variety of laboratory studies (Jennings and Dillehay 2006). In many rat studies, maximizing assay throughput and reducing biological sample volume provides significant advantages, as sample volume is often limited.

Bio-Rad's newly configured Bio-Plex Pro rat cytokine, chemokine, and growth factor assays and rat diabetes assays are magnetic bead–based multiplex assays designed to meet the small sample volume and high-throughput demands of research using rat models. These assays permit the simultaneous measurement of multiple biomarkers in a single sample using as little as 12.5 µl.

The Bio-Plex Pro rat cytokine and rat diabetes sandwich immunoassays are optimized for the Bio-Plex suspension array system and other related platforms using Luminex's xMAP technology. The Bio-Plex suspension array system is more sensitive, has a larger dynamic range, and has a shorter assay time compared to conventional ELISA. The Bio-Plex system can simultaneously quantify up to 100 biomarkers in serum, plasma, cell culture supernatants, and other more exotic sample matrices. These advantages of the Bio-Plex Pro assays and Bio-Plex system translate to reduced labor, improved productivity, and lowered assay cost.

In this work, we have validated the Bio-Plex Pro rat cytokine and rat diabetes assays according to performance criteria of life science, preclinical, and pharmaceutical research. These criteria include assay range, sensitivity, precision, specificity, and linearity of dilution as well as parallelism to evaluate robustness in the key sample matrices mentioned above.

The validation studies covered 25 rat cytokine and 5 rat diabetes markers (Table 1). These markers are available in a 23-plex, a Th1/Th2 12-plex, or 30 singleplex kits for customizable configurations tailored specifically to end-user needs (bulletin 6100).

Table 1. Bio-Plex Pro rat cytokine, chemokine, and growth factor and rat diabetes assay menu.

Rat Assays	23-Plex	Th1/Th2 12-Plex	Singleplex
IL-1α	•	•	•
IL-1β	•	•	•
IL-2	•	•	•
IL-4	•	•	•
IL-5	•	•	•
IL-6	•	•	•
IL-7	•		•
IL-10	•	•	•
IL-12p40			•
IL-12p70	•	•	•
IL-13	•	•	•
IL-17A	•		•
IL-18	•		•
G-CSF	•		•
GM-CSF	•	•	•
GRO/KC	•		•
IFN-γ	•	•	•
M-CSF	•		•
MIP-1α	•		•
MIP-2			•
MIP-3α	•		•
RANTES	•		•
TNF-α	•	•	•
VEGF	•		•
EPO	•		•
Ghrelin			•
GLP-1			•
Glucagon			•
Leptin			•
PAI-1			•

(•) Diabetes markers.

Method

Bio-Plex Pro rat cytokine and rat diabetes assays employ a standard sandwich enzyme immunoassay method using a 96-well plate format. Capture antibodies coupled to fluorescently colored beads are allowed to react with a sample containing proteins of interest. After performing a series of washes to remove unbound analytes, a biotinylated detection antibody specific for a different epitope on the analyte is added to the beads. The result is the formation of a sandwich of antibodies around the specific analyte. The reaction mixture is detected by the addition of the 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 array reader, which identifies and quantifies each specific reaction based on bead color and fluorescent signal intensity (Figure 1).

Analytic Performance Characteristics

Assay Precision

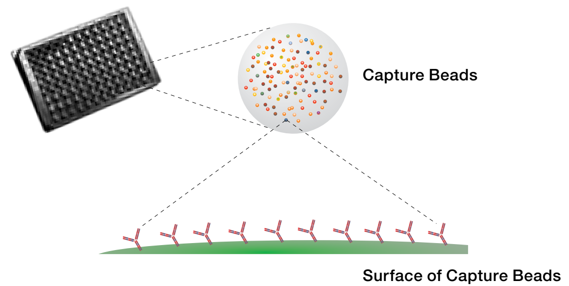
Assay precision includes intra-assay %CV and inter-assay %CV. Intra-assay %CV assesses the variation of median fluorescence intensity (MFI) of the standard points in three replicate wells within a representative assay. In contrast, inter-assay %CV measures the variability of observed standard concentrations among three independent assays. The mean CV was calculated from the standard concentrations within the assay working range (Table 2). The same study was also conducted in RPMI media and showed comparable or better results (data not shown).

Table 2. Assay precision in serum-based standard diluent.

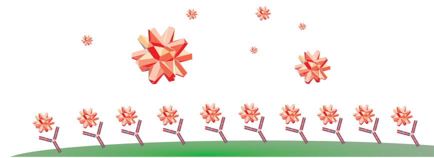
	IL-1α	IL-1β	IL-2	IL-4	IL-5	IL-6	IL-7	IL-10	IL-12p40	IL-12p70	IL-13	IL-17A	IL-18	G-CSF	GM-CSF
Intra-CV%	2.8	3.5	3.6	6.4	4.1	4.3	4.3	3.7	2.2	4.3	3.5	2.9	3.8	2.0	4.4
Inter-CV%	3.4	1.9	2.9	3.2	0.4	2.7	2.7	5.6	4.0	3.4	2.1	1.9	5.4	1.4	1.8

	GRO/KC	IFN-γ	M-CSF	MIP-1α	MIP-2	MIP-3α	RANTES	TNF-α	VEGF	EPO	Ghrelin	GLP-1	Glucagon	Leptin	PAI-1
Intra-CV%	3.0	4.8	2.5	3.7	3.8	3.9	4.4	4.9	2.5	4.3	3.6	7.2	4.1	4.2	5.4
Inter-CV%	2.4	2.9	2.5	6.6	5.2	1.8	2.0	6.7	4.5	1.2	4.5	5.5	3.7	2.9	3.0

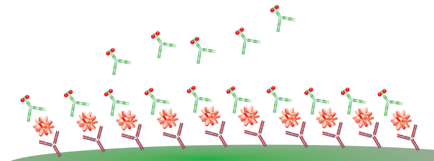
Step One:
Dispense capture beads
Wash plate 2 times



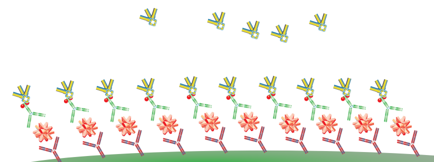
Step Two:
Add samples
Incubate
Wash plate 3 times



Step Three:
Add detection antibody
Incubate
Wash plate 3 times



Step Four:
Add reporter dye
Incubate
Wash plate 3 times



Step Five:
Resuspend beads
Fluorescent sorting
Data analysis

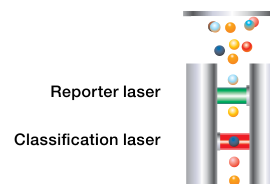


Fig. 1. Schematic representation of a sandwich-based Bio-Plex assay workflow.

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Assay Accuracy

Assay accuracy (recovery) was calculated as the percentage of the observed concentration value of a target antigen relative to the expected value. This was evaluated for each target by determining the standard curve recovery across an eight-point standard curve fitted by a five-parameter logistic regression analysis (Table 3).

Table 3. Standard curve percent recovery in serum and RPMI matrices.

	IL-1α	IL-1β	IL-2	IL-4	IL-5	IL-6	IL-7	IL-10	IL-12p40	IL-12p70	IL-13	IL-17A	IL-18	G-CSF	GM-CSF
Serum	100	99	99	100	101	99	99	100	101	99	99	99	99	99	100
RPMI	99	100	100	100	97	102	100	101	99	100	100	103	99	102	100

	GRO/KC	IFN-γ	M-CSF	MIP-1α	MIP-2	MIP-3α	RANTES	TNF-α	VEGF	EPO	Ghrelin	GLP-1	Glucagon	Leptin	PAI-1
Serum	100	101	99	103	100	98	100	103	99	99	98	99	98	99	100
RPMI	100	99	99	102	101	99	101	99	100	99	102	108	105	100	99

Assay Working Range and Sensitivity

Assay working range is defined as a concentration range between the lowest level of quantification (LLOQ) and the upper level of quantification (ULOQ) in which the assay is both precise (intra-assay replicate precision ≤20%) and accurate (standard recovery within 80–120%). The mean LLOQ and ULOQ from three independent assays were used to derive the overall assay range in a serum-based matrix. Assay sensitivity (limit of detection, LOD) was calculated by adding two standard deviations to the mean background median fluorescence intensity (MFI) of the blank and calculating the corresponding concentration in pg/ml. Table 4 summarizes the findings in both serum and RPMI matrices.

Assay Linearity and Parallelism

Linearity of dilution determines the suitability of a standard curve for reflecting relative quantities of analytes in a given matrix. This was examined by spiking recombinant analyte into rat serum, plasma, or cell culture matrices and performing 1:2 serial dilutions. Each dilution point was assayed in all multiplex assays and representative singleplex assays. The observed and expected analyte concentrations (within assay working ranges) were plotted and the correlation coefficient (R^2) values were generated from linear regression analysis.

Parallelism is a second measure of how well an assay's standard curve and the measured analyte in key sample matrices correlate across the range of the assay. If the standard curve is a reasonable predictor of target levels in a given sample, then the standard curve should be parallel to a curve drawn for a range of target quantities measured in the sample. In this study, assay parallelism was investigated by measuring slope differences between spike response curves in rat serum or plasma and the standard curve in standard diluent. Spiked standard diluent served as a positive control to indicate best-case results. Overall, dose-response curve slopes tended to be ≤30% different for spiked serum and plasma. The exception is PAI-1, which reported a >30% difference in serum due to its high endogenous levels (Table 5).

Table 4. Assay working range and assay sensitivity (pg/ml).

	Serum		RPMI*		Assay Sensitivity	
	LLOQ	ULOQ	LLOQ	ULOQ	Serum	RPMI*
IL-1α	2.0	24,649	2.0	32,865	1.0	0.8
IL-1β	5.8	31,666	7.7	31,666	1.8	1.0
IL-2	7.8	7,981	7.8	7,981	2.6	1.9
IL-4	1.0	16,209	1.0	16,209	1.0	0.2
IL-5	5.9	2,007	2.0	8,030	6.5	2.2
IL-6	13.1	30,766	7.5	30,766	9.7	3.5
IL-7	2.0	32,472	2.0	32,472	0.4	1.6
IL-10	4.2	34,385	8.4	34,385	4.9	3.6
IL-12p40	2.0	2,015	2.0	8,058	0.4	0.6
IL-12p70	3.8	15,481	1.9	30,961	0.7	1.5
IL-13	1.9	15,751	1.9	31,502	0.9	0.7
IL-17A	1.0	3,079	4.0	4,105	0.1	0.2
IL-18	3.9	31,652	3.9	15,826	3.6	1.3
G-CSF	1.0	2,911	1.0	3,881	0.2	0.2
GM-CSF	5.8	31,519	1.9	31,519	0.6	0.7
GRO/KC	2.0	1,521	2.0	2,028	0.6	0.2
IFN-γ	3.9	23,955	2.0	31,940	1.2	0.9
M-CSF	1.9	23,264	1.9	31,019	0.4	0.6
MIP-1α	25.3	4,325	8.5	8,651	11.9	3.4
MIP-2	0.3	1,085	0.3	1,085	0.2	0.0
MIP-3α	1.0	3,032	1.0	1,011	0.7	0.0
RANTES	7.8	7,950	1.9	7,950	3.1	1.4
TNF-α	16.0	8,207	128.2	8,207	2.9	1.3
VEGF	0.5	3,963	0.5	7,925	0.3	0.2
EPO	31.5	32,258	7.9	32,258	7.9	2.0
Ghrelin**	1.0	16,152	16.0	16,152	0.3	1.0
GLP-1**	4.0	6,062	32.0	2,021	3.3	3.0
Glucagon**	5.9	4,443	6.0	1,481	5.7	2.0
Leptin**	23.9	130,465	32.0	130,465	5.3	5.0
PAI-1**	48.7	66,888	65.0	66,888	27.0	69.0

* Only one representative assay was used to derive values in the RPMI matrix.
** The diabetes markers were evaluated in a separate multiplex.

Assay Specificity (% Cross-Reactivity)

Assay specificity was examined by subjecting specific test reagents to single-antigen and single-detection cross-reactivity studies. The single-antigen study evaluates the specificity of a capture antibody. This is conducted by testing an individual antigen in the presence of multiplexed capture beads and detection antibodies. The single-detection study evaluates the specificity of the detection antibodies. This is conducted by testing the individual detection antibody in the presence of multiplexed antigens and capture beads. Overall the studies showed that all assays were highly specific at <5% cross-reactivity (data not shown). The exceptions were IL-12p70, IL-12p40, MIP-2, and GRO/KC. The IL-12p40 capture and detection antibodies cross-react with the IL-12p70 antigen, which contains both p40 and p30 subunits. The capture and detection antibodies of GRO/KC cross-react with the MIP-2 antigen, which shares 50% homology in amino acid sequence with GRO/KC. These results suggest that both IL-12p40 and MIP-2 should be tested as singleplex assays.

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Table 5. Linearity of dilution and parallelism analysis.

	Linearity (R^2)				Parallelism, % Difference		
	Standard Diluent	Serum	Plasma	RPMI	Standard Diluent	Serum	Plasma
IL-1α	1.000	0.999	1.000	0.996	3.8	11.3	12.4
IL-1β	0.999	0.999	1.000	0.998	20.2	8.8	13.4
IL-2	0.999	0.999	1.000	0.996	8.1	17.8	11.2
IL-4	1.000	0.995	0.994	0.997	4.9	9.3	11.7
IL-5	0.995	0.992	0.987	0.982	0.4	15.1	1.2
IL-6	0.999	0.961	0.999	0.998	8.2	15.1	9.1
IL-7	1.000	0.992	0.999	0.992	0.1	29.7	23.2
IL-10	0.999	0.991	0.999	0.989	0.7	16.8	3.5
IL-12p40	0.999	1.000	1.000	0.998	3.0	19.4	3.0
IL-12p70	1.000	0.998	1.000	0.998	8.9	18.9	2.3
IL-13	1.000	0.999	0.999	0.997	2.0	5.4	18.4
IL-17A	0.999	0.999	1.000	0.999	2.3	10.4	24.5
IL-18	0.999	1.000	0.999	0.999	14.4	24.2	19.9
G-CSF	0.989	1.000	0.999	0.998	8.1	13.9	12.1
GM-CSF	1.000	0.999	0.995	0.999	0.5	21.9	13.2
GRO/KC	1.000	1.000	0.997	0.987	3.9	29.6	9.2
IFN-γ	1.000	0.999	0.999	0.996	9.2	18.5	3.2
M-CSF	1.000	0.999	1.000	0.999	1.3	31.6	0.4
MIP-1α	0.999	0.999	0.996	0.996	18.9	0.6	35.0
MIP-2	0.994	0.994	0.999	0.994	22.6	26.6	25.8
MIP-3α	0.999	1.000	1.000	0.996	7.5	20.3	0.0
RANTES	0.995	0.981	1.000	0.992	6.0	27.3	19.2
TNF-α	0.995	0.996	0.996	0.996	0.8	7.2	8.3
VEGF	1.000	0.999	0.999	0.997	2.5	7.7	11.2
EPO	0.999	0.989	0.999	0.997	12.1	9.1	8.4
Ghrelin	0.997	0.998	0.994	–	14.8	19.7	28.9
GLP-1	1.000	0.989	1.000	–	29.2	20.4	7.7
Glucagon	0.991	1.000	1.000	–	14.2	32.5	18.0
Leptin	0.998	1.000	0.999	–	15.3	0.3	3.4
PAI-1	1.000	0.991	0.996	–	30.2	51.4	15.5

(–) Data not available.

Table 6. Percent of samples detected within working assay range.

	Samples Detected within Assay Range, %		
	Normal (n=13)	LPS-treated (n=10)	Total (n=23)
IL-1α	100	100	100
IL-1β	100	100	100
IL-2	100	100	100
IL-4	100	100	100
IL-5	100	100	100
IL-6	100	100	100
IL-7	100	100	100
IL-10	100	100	100
IL-12p40	100	100	100
IL-12p70	92	100	96
IL-13	92	100	96
IL-17A	100	100	100
IL-18	100	100	100
G-CSF	100	100	100
GM-CSF	92	100	96
GRO/KC	92	100	96
IFN-γ	100	100	100
M-CSF	100	100	100
MIP-1α	69	70	70
MIP-2	100	100	100
MIP-3α	100	100	100
RANTES	100	100	100
TNF-α	92	100	96
VEGF	100	100	100
EPO	100	100	100
Ghrelin	100	100	100
GLP-1	100	100	100
Glucagon	100	100	100
Leptin	100	100	100
PAI-1	100	100	100

Specimen Testing

Samples from key matrices (serum and plasma) were tested to examine the robustness of the assay ranges. The assays were able to measure the majority of samples within the specified working assay range (Table 6).

The expected trends in pooled normal versus disease-state samples were evident in a set of single-dose lipopolysaccharide (LPS)-treated rats, in which higher levels of cytokines were detected in the majority of markers in both plasma and serum matrices (Table 7).

Table 7. Cytokine levels (pg/ml) of healthy and LPS-challenged rat serum and plasma.

	IL-1α	IL-1β	IL-2	IL-4	IL-5	IL-6	IL-7	IL-10	IL-12p40	IL-12p70	IL-13	IL-17A	IL-18	G-CSF	GM-CSF
Normal plasma	42	119	73	22	59	325	44	263	12	OOR*	OOR	9	400	5	OOR
LPS plasma	231	1,636	382	140	219	9,827	246	3,841	39	118	121	36	531	20	161
Normal serum	831	2,369	1,318	827	676	9,077	2,997	3,185	117	536	460	141	2,071	73	860
LPS serum	2,201	7,211	3,140	1,904	1,055	25,283	4,245	8,974	381	1,728	1,022	332	3,732	227	2,490

	GRO/KC	IFN-γ	M-CSF	MIP-1α	MIP-2	MIP-3α	RANTES	TNF-α	VEGF	EPO	Ghrelin	GLP-1	Glucagon	Leptin	PAI-1
Normal plasma	OOR	32	191	OOR	4	17	212	OOR	23	2,317	2,860	50	126	2,117	OOR
LPS plasma	3,578	264	476	2,395	398	50	874	11,173	70	2,093	3,589	158	221	1,756	3,096
Normal serum	317	859	571	912	148	172	3,625	1,449	285	4,786	857	294	220	4,162	1,822
LPS serum	3,734	2,639	1,130	4,362	2,218	343	4,647	11,084	526	6,617	3,071	726	426	6,393	4,243

* OOR = out of operating range.

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Agreement with Other Luminex Platforms

To give end users the flexibility of using other Luminex platforms, the rat assays were also evaluated on both the Luminex FlexMAP 3D and MAGPIX systems. Samples tested (n=14) included normal and diseased plasma and serum. The Bio-Plex 200 platform closely matched both the FlexMAP 3D and the MAGPIX systems in sample readout, as reported in the percentage of detectable samples within the working assay range (Table 8). The exceptions are GM-CSF, GRO/KC, and GLP-1. These discrepancies were due to minor fluctuations in standard recovery that resulted in certain sample measurements falling out of assay range. Overall, the MFI values are comparable between the Bio-Plex 200 and MAGPIX systems. The MFI values obtained from FlexMAP 3D were higher due to its enhanced PMT setting. However, sample concentrations generated with these three instruments were comparable.

Table 8. Samples detected within the assay range on different sytems.

	Samples in Range, % (n=14)		
Target	**Bio-Plex System**	**MAGPIX**	**3D**
IL-1α	100	100	100
IL-1β	100	100	100
IL-2	100	100	71
IL-4	100	100	100
IL-5	93	100	100
IL-6	100	100	100
IL-7	100	100	85
IL-10	100	100	100
IL-12p40	100	100	100
IL-12p70	100	100	96
IL-13	100	100	96
IL-17A	100	100	100
IL-18	100	100	100
G-CSF	100	100	100
GM-CSF	100	71	96
GRO/KC	78	100	78
IFN-γ	100	100	100
M-CSF	100	100	100
MIP-1α	78	85	85
MIP-2	100	100	100
MIP-3α	100	100	100
RANTES	100	100	100
TNF-α	92	100	100
VEGF	100	100	100
EPO	100	100	100
Ghrelin	100	100	100
GLP-1	57	100	100
Glucagon	100	100	100
Leptin	100	100	100
PAI-1	100	100	100

Conclusions

The validation studies presented here demonstrate the robustness of the 25 rat cytokine and 5 rat diabetes assays. Assay precision, accuracy, working range, sensitivity, linearity, parallelism, and specificity were all evaluated. Additionally, the relevance of the assay working ranges and the equivalence of the Bio-Plex suspension array system to other Luminex platforms were confirmed using specimen testing.

References

Jennings VM and Dillehay DL (2006). Immunology. In The Laboratory Rat, second edition, Suckow, MA, Weisbroth, SH, Franklin, CL eds. (Academic Press), pp. 847–864.

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