Selective and robust biomarker quantification using Gyrolab human cytokine kit reagents

Application Note D0043944/A

Introduction:

The use of biomarkers in biotherapeutic development to monitor drug pharmacodynamics, safety, and efficacy has been steadily increasing in an FDA-supported effort to guide decision making and improve the success of new therapeutics in clinical studies. Context of use for these assays demands high performance and reliability, which typically points to single-analyte assays over multiplex screening assays to enable straightforward validation and assay robustness.

Recently, single-analyte assay kit reagents (CD labware sold separately) for the quantification of cytokines IL-4, IL-6, IL-10, IFN-gamma, and TNF-alpha in human and cynomolgus monkey (cyno) serum were introduced for the Gyrolab® immunoassay automated microfluidic platform. This format offers advantages of speed and walkaway time over plate-based immunoassays including ELISA which require extensive manual pipetting and hands-on time. In addition, the platform offers a 5-CD configuration allowing continuous runs of different assays on each CD in series ("Gyroplex™ panel") a convenient approach for multi-analyte analysis.

In addition to performance metrics of sensitivity and dynamic range, cytokine quantification requires discrimination of the analyte of interest in biological matrices containing other biomolecules. In this application note, the detection of 5 human cytokines, IL-4, IL-6, IL-10, IFN-gamma, and TNF-alpha in single-analyte assays using the Gyrolab Cytokine Kit Reagents and Gyrolab platform was investigated in human and cyno serum samples spiked with the other four cytokines to evaluate the selectivity and robustness of the assays in the presence of the other cytokines. The assay ranges, spike recovery, and dilutional linearity were measured to assess the assay performance for each of the 5 cytokine analytes.

Materials and Methods

Samples and Gyrolab Cytokine Kit Reagents

Gyrolab Cytokine Kit Reagents for human IL-4, IL-6, IL-10, TNF-alpha, and IFN-gamma containing capture, and detect reagents, stock standard, wash buffer, and sample dilution buffer were used with the recommended Gyrolab Bioaffy™ 4000 CD and Gyrolab method found at https://www.gyrosproteintechnologies.com/immunoassays/products/gyrolab-kits-solutions#Biomarkers

All 5 cytokine assays follow the same sandwich assay format (Figure 1) with biotinylated anti-human cytokine monoclonal antibody capture reagent and an Alexa Fluor® 647 labeled anti-human cytokine monoclonal antibody detection reagent.

Gyrolab immunoassays are performed with the biotinylated capture reagent attached to streptavidin-coated beads in a microfluidic flow-through 15-nL affinity column within a CD-based labware. Reagent and sample volumes are determined by volume definition chambers and are not subject to pipetting errors, and liquid flow carefully controlled using centrifugal forces within the automated platform. Fluorescent reads are also automated, with the added bonus of scans across the column generating column profile data available for QC analysis of every data point, unlike any other immunoassay technology.

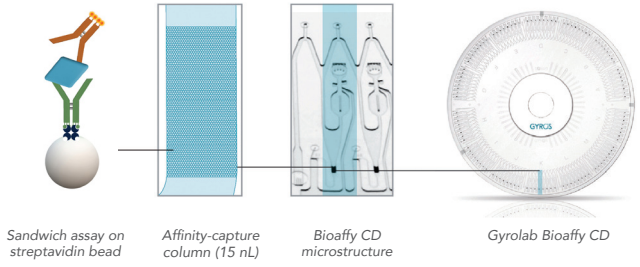


Figure 1. Cytokine analysis sandwich assay format on the Gyrolab microfluidic platform. The human and cyno cytokine assays for IL-4, IL-6, IL-10, IFN-gamma, and TNF-alpha were all three-step sandwich immunoassays with biotinylated anti-human cytokine monoclonal antibody capture reagent and an Alexa Fluor® 647 labeled anti-human cytokine monoclonal antibody detection reagent. Recombinant human cytokine was used as standard material.

Samples for dilutional linearity and selectivity

Human and cyno serum samples with known low endogenous levels were used to prepare experimental samples spiked with recombinant human cytokines. In order to measure the Gyrolab cytokine assay performance in the presence of the other cytokines, two human serum samples and two cyno serum samples were prepared with different concentrations of all five cytokines analyzed in duplicate in this study (Table 1). The different spike combinations evaluated different relative cytokine levels to look for effects on analysis results.

Samples were diluted using the MRD of each assay (Table 2) and subsequently serially diluted 1:2 four times in Biomarker Sample Dilution Buffer 1. Of note, human IL-10 was not measured in the cyno serum since this assay does not have cyno cross-reactivity.

Instrument

The 5 cytokine assays were analyzed on separate Gyrolab Bioaffy 4000 CDs with different assays on each CD in one continuous 5-CD run (Gyroplex panel) on a Gyrolab xPand, with on-deck cooling to preserve reagents, samples and standards.

Results and Discussion

Standard curves and assay performance

Standard curves for all assays are shown in Figure 2. Published assay ranges and recommended MRD for the Gyrolab Cytokine Kit Reagents used for the assays are shown in Table 2.

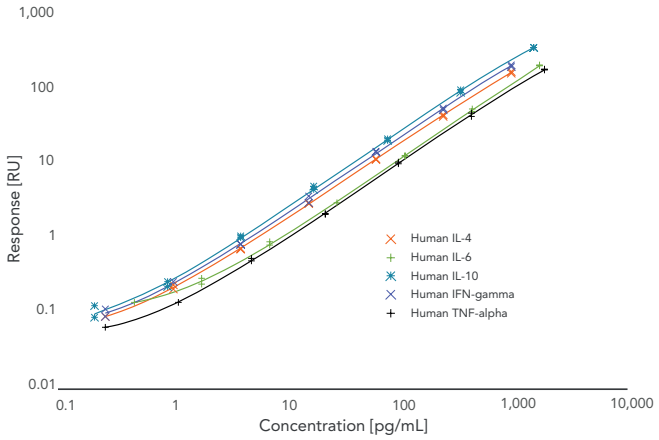


Figure 2. Standard curves of human IL-4, IL-6, IL-10, TNF-alpha, and IFN-gamma Gyrolab assays. Samples were run in duplicate with each assay on a separate CD run sequentially in a 5-CD Gyroplex format on a Gyrolab xPand.

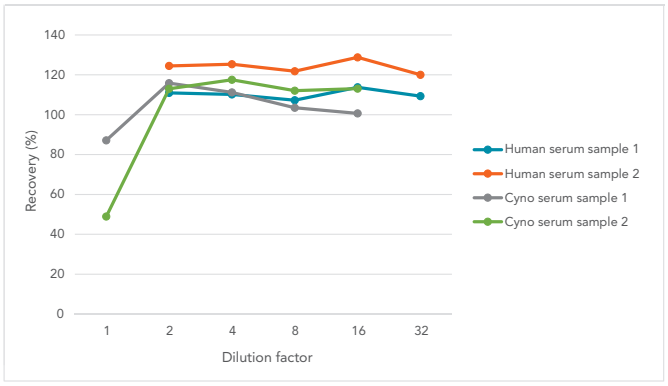
Table 1. Spike combinations for cytokine experimental samples. The different spike combinations were utilized to evaluate the selectivity and dilutional linearity of the Gyrolab Human Cytokine Kit Reagent assays with the other cytokines present in high and low concentrations within the analytical range of the assay.

Analyte	Spike combination 1		Spike combination 2		Spike combination 3		Spike combination 4	
	Expected conc in neat serum (pg/mL)	Actual measured) conc (pg/mL)	Expected conc in neat serum (pg/mL)	Actual measured conc (pg/mL)	Expected conc in neat serum (pg/mL)	Actual measured conc (pg/mL)	Expected conc in neat serum (pg/mL)	Actual measured conc (pg/mL)
Human IL-4	140	137	40	34	40	38	140	131
Human IL-6	180	197	180	222	180	195	180	202
Human IL-10	160	167	40	44	40	43	40	42
Human IFN-gamma	140	138	40	40	40	38	140	137
Human TNF-alpha	200	191	200	202	200	210	200	191

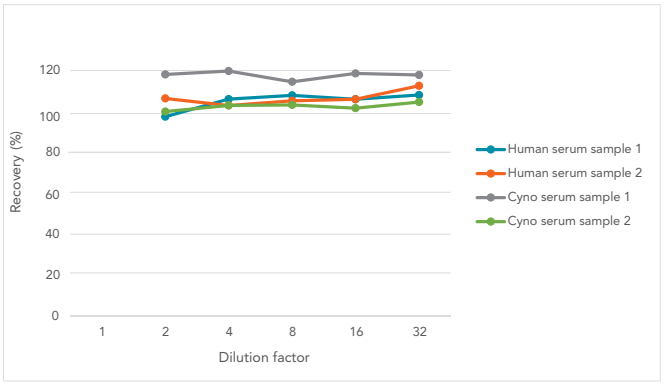
The recovery (found in Figure 3) of the serum samples are calculated based on the calculated concentrations (right column for each spike combination).

Table 2. Assay performance and recommended minimum required dilution (MRD) for Gyrolab Cytokine Kit Reagents. LOD (Limit of Detection) was determined as two standard deviations (SD) above the blank. Lower Limit of Quantitation (LLOQ) and Upper Limit of Quantitation (ULOQ) were determined by analyzing QC samples from six runs by two operators over two days in the lower and upper regions of the assay's analytical range, respectively. See Gyrolab® Human Cytokine Kit Reagents Product Information Sheet for experimental details.

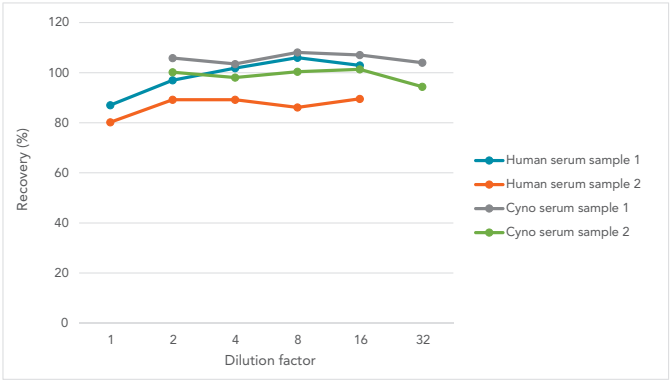
Analyte	LOD (pg/mL)	LLOQ (pg/mL)	ULOQ (pg/mL)	MRD Human Serum	MRD Cyno Serum
Human IL-4	<0.5	~0.8	~800	2	1
Human IL-6	<1.5	~2	~1400	1	2
Human IL-10	<0.3	~0.75	~1200	1	N/A
Human IFN-gamma	<0.4	~1	~800	2	2
Human TNF-alpha	<0.7	~1	~1500	2	1



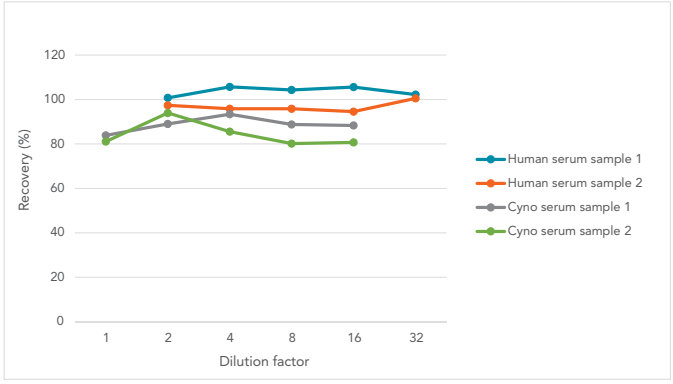
Human IL-4



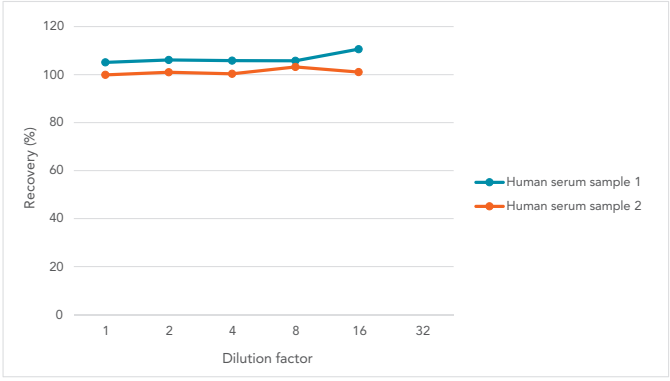
Human IFN-gamma



Human IL-6



Human TNF-alpha



Human IL-10

Figure 3. Dilutional linearity for human and cyno experimental samples using Gyrolab Cytokine Kit Reagents. Single-analyte assays were run on samples containing a mixture of the 5 cytokines.

Specificity, spike recovery, and linearity

The standard curves for the Gyrolab human cytokine assays for IL-4, IL-6, IL-10, IFN-gamma, and TNF-alpha show a wide dynamic range and excellent sensitivity to low- or sub-pg/mL levels with 3 logs quantifiable range as shown in Table 2 and Figure 1. Excellent spike recovery and dilutional linearity were shown for the experimental samples prepared as a mixture of all 5 cytokines for all the cytokine assays (Table 3 and Figure 3). One sample, cyno serum sample 2 IL-4 quantification with spike mix 4, showed poor recovery of 54%, while the other samples in the same dilution series showed complete recovery (80-130%). One data set, quantification of IL-4 in spike combination 2, produced recovery >120%.

Excluding the data point above, all other data across all assays showed a maximum of 129% recovery and minimum of 80%, with a mean recovery of 102% and a standard deviation (SD) of 10. This mean and SD recovery demonstrates excellent matrix tolerance and assay robustness.

Assay robustness

The assay results were exceptionally precise, with a mean %CV of 3.9, SD 3.9. Except for one %CV of 21.5, all other precision data was 17.5% or below. This high precision is characteristic of Gyrolab platform assays due to the robustness and reproducibility of the microfluidic assay design, and these results demonstrate that the combination of the Gyrolab Human Cytokine Kit Reagents, the Gyrolab Bioaffy 4000 CD, and Gyrolab platform is well-suited for cytokine quantification.

Automated Gyroplex analysis of 5 cytokines

All 5 cytokine assays using the Gyrolab Human Cytokine Kit Reagents were completed in an automated, walkaway run with a total run time of ~8 h 15 min, or approximately 90 minutes per CD assay, with sample preparation time typically 30-60 minutes. Gyrolab xPand 5-CD runs can be completed overnight or during a normal workday, with on-deck sample cooling possible to minimize sample evaporation or degradation.

Running Gyrolab single-analyte assays in series on separate CDs (Gyroplex panel) is a time- and resource-conserving approach to generate high-quality biomarker data from serum samples. This approach, and the use of Gyrolab CD-based single-analyte assays has important advantages in biomarker analysis:

- Single-analyte assays eliminate cross-talk or reagent interference since each data point is generated from a dedicated, isolated microstructure on the Gyrolab Bioaffy CD
- Well-optimized and individually tailored assays provide reliable and reproducible assay data
- Each assay can be individually validated and validation is straightforward without interference from other analytes
- There is no need to rerun all assays if one fails

Table 3. Cytokine analysis of human and cyno serum experimental samples. Five dilutions were run for each analyte and sample. CV concentration values of "-" represents where one replicate was excluded as an outlier so no CV value was calculated.

Sample	Dilution	Human IL-4			Human IL-6			Human IL-10			Human IFN-gamma			Human TNF-alpha		
		Average back calculted conc. (pg/mL)	Recovery (%)	CV conc (%)	Average back calculted conc. (pg/mL)	Recovery (%)	CV conc (%)	Average back calculted conc. (pg/mL)	Recovery (%)	CV conc (%)	Average back calculted conc. (pg/mL)	Recovery (%)	CV conc (%)	Average back calculted conc. (pg/mL)	Recovery (%)	CV conc (%)
Human serum sample 1 (spike combination 1)	1:1	N/A			172	87	3.0	176	105	5.3	N/A			N/A		
	1:2	152	111	9.1	191	97	6.3	177	106	1.2	130	94	0.5	192	101	2.3
	1:4	151	110	1.8	201	102	2.8	177	106	2.5	139	101	5.7	202	106	3.8
	1:8	147	107	0.1	209	106	5.7	177	106	3.9	141	102	8.2	199	104	0.2
	1:16	156	114	12	203	103	2.6	185	111	1.3	139	101	1.9	201	106	-
	1:32	150	109	2.8	N/A			N/A			141	102	5.0	195	102	1.4
Human serum sample 2 (spike combination 2)	1:1	N/A			178	80	8.0	44	100	3.2	N/A			N/A		
	1:2	42	124	4.0	198	89	0.2	45	101	1.2	40	101	0.6	197	97	5.6
	1:4	42	125	8.0	198	89	3.0	44	100	1.0	39	98	-	194	96	2.2
	1:8	41	122	6.7	191	86	0.7	46	103	2.5	40	100	4.8	194	96	-
	1:16	44	129	1.3	199	90	2.8	45	101	-	40	101	1.0	191	95	6.0
	1:32	41	120	4.8	N/A			N/A			42	105	-	203	101	1.8
Cyno serum sample 1 (spike combination 3)	1:1	33	87	7.0	N/A			N/A			N/A			176	84	2.5
	1:2	44	116	-	206	106	1.2				42	111	1.4	187	89	0.3
	1:4	43	111	5.4	202	103	0.1				43	112	1.1	196	93	3.1
	1:8	40	104	9.9	211	108	4.3				41	108	5.7	187	89	2.8
	1:16	39	101	3.1	209	107	5.4				42	111	4.9	186	88	2.6
	1:32	N/A			203	104	-				42	111	-	N/A		
Cyno serum sample 2 (spike combination 4)	1:1	64	49	17	N/A			N/A			N/A			155	81	11
	1:2	148	113	8.3	203	100	4.0				131	96	3.7	179	94	2.1
	1:4	154	118	0.1	198	98	1.9				134	98	1.9	163	86	5.0
	1:8	146	112	1.3	203	100	2.3				135	99	1.6	153	80	5.4
	1:16	148	113	0.5	205	101	7.0				133	97	2.2	154	81	2.3
	1:32	N/A			191	94	22				136	100	1.2	N/A		

Summary and conclusions

The assay specificity for analysis of single human cytokines (IL-4, IL-6, IL-10, IFN-gamma, and TNF-alpha) in human and cyno serum samples containing all 5 of the cytokines was demonstrated using the recently introduced Gyrolab Human Cytokine Kit Reagents on the Gyrolab xPand. Spike recovery, linearity, and assay precision were excellent, showing tolerance towards human and cyno serum matrix and robust performance of the kit reagents in the presence of other cytokines.

Gyrolab microfluidic immunoassay platform, a trusted high-performance assay technology by pharma and biotech for over 15 years, provides a reliable, precise, and fast automated approach to biomarker measurement. Sensitivity of Gyrolab immunoassays for biomarker analysis has been recently improved through the introduction of the Gyrolab Bioaffy™ 4000 CD, extending quantification levels to low pg/mL or high fg/mL levels, and use of Gyrolab platform for biomarker assays has increased with the introduction of this new CD.

Gyrolab assays are well-suited for biomarker analysis during biotherapeutic development for applications such as pharmacodynamic, safety, or surrogate endpoint biomarker use where high sensitivity, matrix tolerance, wide dynamic range, and assay robustness is suitable for validation and regulated environments.

The introduction of the Gyrolab Human Cytokine Kit Reagents provides a ready-to-use assay solution for analysis of cytokines IL-4, IL-6, IL-10, IFN-gamma, or TNF-alpha in human or cyno serum suitable for regulated environments and use in development programs to guide decision making in preclinical or clinical studies.

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