Performance Characterization of Ultra-Sensitive Cytokine Detection Immunoassays

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Abstract:

Purpose: Ultra-sensitive, subpicogram immunoassay platforms enable detection and quantitation of endogenous low abundance biomarkers. Four single molecule counting assays (IL6, IL17F, IL17A and IL1β) were characterized for precision, specificity, linearity of dilution, spike recovery and effective quantitation of endogenous levels.

Methods: Singulex® IL6, IL17F, IL17A and IL1β assay kits were evaluated. Lower limits of quantitation (LLOQ) and limits of detection (LOD) were calculated from standard curves. Quantitation of endogenous levels in commercially sourced normal human sera and plasma was performed. Inter-assay precision of endogenous analytes was determined using distinct runs. Specificity was assessed by signal reduction using polyclonal antibodies (PAbs) or exchanging capture microparticles for different analytes. Linearity of dilution and spike recovery was assessed in normal human serum and/or plasma.

Results: LOD values were 0.015, 0.700, 0.020 and 0.040 pg/ml for IL6, IL17F, IL17A and IL1β, respectively. LLOQ values were 3 to 10-fold higher than LOD. Median quantifiable IL6 levels were 1.74 (n = 21) and 0.97 pg/ml (n = 15) in serum and plasma, respectively. IL17F quantifiable levels were 9.7 (n = 34) and 12.0 pg/ml (n = 15), respectively. Median quantifiable IL1β levels were 0.72 pg/ml for serum (n = 18), excluding 3 undetectable and 3 samples <LLOQ. Median quantifiable plasma levels were 0.115 pg/ml (n = 14, 1 sample <LLOQ excluded). Serum IL17A (n = 18) was generally not quantifiable, with one sample >LLOQ, 16 >LOD and 2 undetectable. Median detectable IL17A levels were 0.08 and 0.04 pg/ml for serum and plasma (n = 12), respectively. Average inter-assay precision ranged from 11.8% to 22.3% CV, and average spike recovery was 74.4% to 93.1%. Specificity was demonstrated for antigen standard in the presence of competing PAbs or alternative microparticles, with variability noted in serum and plasma depending upon LLOQ and PAb effectiveness.

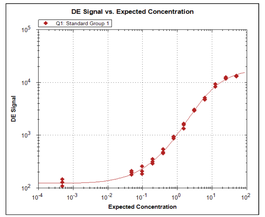
Conclusion: These evaluations confirm that subpicogram analyte levels can be detected and often quantified in normal human serum and plasma. A firm handle on limits of detection and quantitation, as well as specificity, was acquired to assess fit-for-purpose characteristics.

Introduction:

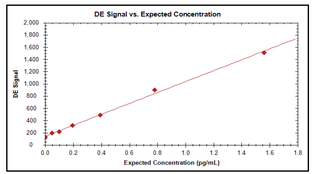
Measurement of low abundance biomarkers has been challenging due to a lack of available assays with suitable sensitivity. This has hampered efforts by researchers to effectively study disease states and the effect of treatments. Recently, several new immunoassay technologies have become available to allow for quantitation of these low-abundance molecules. The Singulex Erenna® platform utilizes proprietary single molecule counting technology in conjunction with robust microparticle-based sandwich immunoassays to enable detection and quantitation of analytes at femtogram/ml concentrations. A dynamic range of over 4 logs allows detection at previously unprecedented levels in undiluted complex matrices, such as serum and plasma. We examined the sera and plasma from normal healthy donors to characterize four Erenna® assays: IL-6, IL-17A, IL-17F and IL-1β. Assays were evaluated for limits of detection and quantitation, inter-assay precision of endogenous levels and specificity of the assay signal. Matrix effects were evaluated using linearity of dilution studies and spike recovery.

Materials and Methods.

All sera and plasma samples were obtained from Bioreclamation Inc. East Meadow, NY. Assay kits and related reagents were obtained from Singulex (Alameda, CA). Data were analyzed in SgxLink (Singulex) and Prism GraphPad Software (La Jolla, CA). LLOQ was determined as the concentration at which interpolated analyte recovery was within 20% of the expected value and CV ≤ 20%. LOD was determined as 2 standard deviations above the background divided by the slope of the linear portion of the standard curve. Inter-assay precision was calculated from sample analyte quantitation on distinct days. Spike recovery was calculated by adding endogenous concentration from interpolated results of spikes and determining % of expected recovery. Linearity of dilution was performed by diluting samples with known endogenous levels in supplied standard curve diluent, followed by determination of expected recovery. Specificity studies utilizing antibody were performed by pre-incubation of samples with antibody for 1 hour prior to sample clarification. Specificity was also conducted by testing antigen standard in the presence of microparticles complexed to non-antigen antibody from a distinct assay kit. Samples were clarified by filtration prior to assay, as per kit protocols. All data are the average of 2-3 replicates.



Full Standard Curve for IL-1β

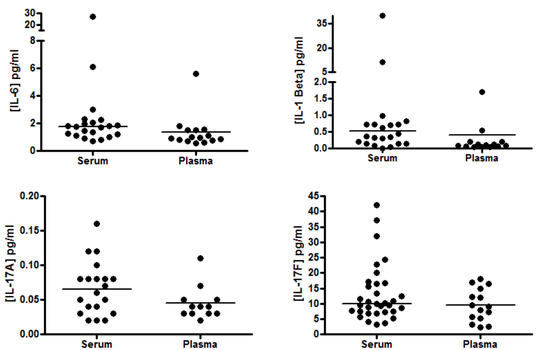


High-Sensitivity portion of IL-1β Standard Curve

DE = Detected Events

Assay	LOD (pg/ml)	LLOQ (pg/ml)	Matrix Compatibility	Median Endogenous Level (pg/ml) Normal Healthy Donors
IL-6	0.015	0.10	Serum Plasma	1.74 0.97
IL-17F	0.700	2.40	Serum Plasma	9.7 12.0
IL-17A	0.020	0.20	Serum Plasma	0.08* 0.04*
IL-1β	0.040	0.20	Serum Plasma	0.72 0.12

**Majority of IL-17A Samples >LOD, but <LLOQ*



Inter-Assay Precision and Spike Recovery

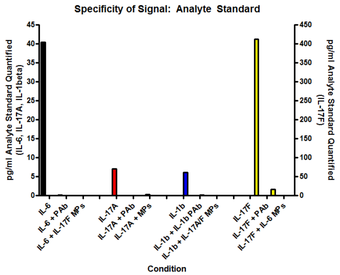
Assay	Inter-Assay Precision (average % CV) Normal Healthy Donors	Spike-Recovery (average %) Normal Healthy Donors
IL-6	11.8%^{S}	74.4%^{S}
IL-17F	13.8%^{S}	83.2%^{S}
***IL-17A**	*22.3%^{S}	81.0%^{S}
IL-1β	13.2%^{S} 16.7%^{P}	93.1%^{S} 90.8%^{P}

S = Serum; P = Plasma
**Majority of IL-17A Samples >LOD, but <LLOQ*

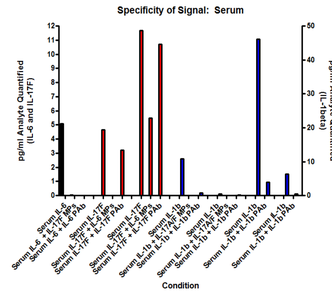
IL-17A Concentration (pg/ml)	Observed (pg/ml)	Expected (pg/ml)	% Recovery
Endogenous – Donor 1	0.12	--	--
75 pg/ml	47.88	75.12	63.7
3 pg/ml	2.32	3.12	74.4
		Mean	*69.1*
Endogenous – Donor 2	0.06	--	--
75 pg/ml	65.16	75.06	86.8
3 pg/ml	3.55	3.06	116.0
		Mean	*101.4*
Endogenous – Donor 3	0.08	--	--
75 pg/ml	54.33	75.08	72.4
3 pg/ml	2.82	3.08	91.6
		Mean	*82.0*
Endogenous – Donor 4	0.08	--	--
75 pg/ml	60.89	75.08	81.1
3 pg/ml	2.89	3.08	93.8
		Mean	*87.4*
Endogenous – Donor 5	0.04	--	--
75 pg/ml	43.89	75.04	58.4
3 pg/ml	2.37	3.04	78.0
		Mean	*68.2*

Spike Recovery for IL-17A:
Spike Recovery was performed by adding known concentration of IL-17A to healthy donor serum.

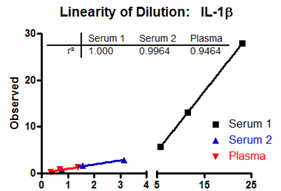
Overall Mean Recovery = 81.6%

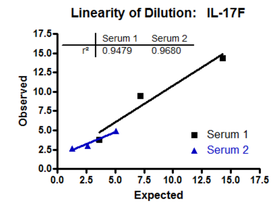


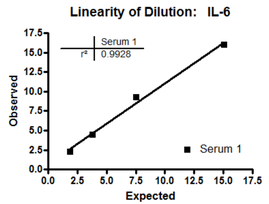
Specificity of signal assessed using a high concentration of analyte standard ± PAb to analyte or alternative kit microparticles
Signal is highly selective for antigen



Serum + alternative MPs or PAbs.
PAbs not previously tested for efficacy in serum or plasma
Signal knock-down confirmed







Linearity of Dilution:
Linearity of dilution performed by diluting samples with endogenous analyte in kit diluent.

R^2 Values ≥ 0.94

Conclusions:

IL-6, IL-17F, IL-17A and IL-1β assays provide accurate and consistent analyte quantitation in normal human sera and plasma. Assays demonstrated specificity of signal and dilutional linearity. The ability to reproducibly quantify sub-picogram levels of analyte in undiluted human sera and plasma with little matrix effect enables unprecedented biomarker and cytokine evaluation.