Conferma™ Human IL-6 ELISA kit
Verification Summary

Analyte: IL-6

Species: Human

Kit Catalog # EZIL6-98K

For Research Use Only. Not for use in diagnostic procedures.

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EMD Millipore Corporation
3050 Spruce Street
St. Louis, Missouri 63103
United States of America
emdmillipore.com

Contents

1. Assay Summary Results

Lower Limit of Quantification (LLOQ)	1.17 pg/mL
Upper Standard Curve Limit	150 pg/mL
Total Samples Tested	30 Normal samples and 30 diseased samples
Endogenous Sample Range	Serum 0 – 7.2 pg/mL, Avg 2.3 pg/mL Plasma 0- 22 pg/mL, Avg 3.8 pg/mL
Spike Recovery Range	Serum: 91 – 107% Plasma: 96 – 109%
Spike Recovery Mean	Serum: 97% Plasma: 103%
Linearity Range Tested	1:2, 1:4, 1:8
Linearity Range	Serum: 105 – 116% Plasma: 88 – 105%
Linearity Mean	Serum: 110% Plasma: 97%
Inter-assay CV% (five samples on 3 plates, by 3 operators)	Mean CV 7.5%
Standard Curve	1.17 – 150 pg/mL
Sample Volume	50 µL neat
Species	Human
Matrix	K2 EDTA Plasma, Serum

2. Kit Protocol

The Conferma™ Human IL-6 ELISA Kit EZIL6-98K was run according to the package insert.

Subsequent revisions of the kit and/or the package insert may have occurred or will occur. These changes are immaterial to the presented experimental data.

Data was analyzed using Belysa™ Software.

3. Representative Standard Curve

The range is from 1.17 pg/mL to 150 pg/mL, with a lower limit of quantification (LLOQ) of 1.17 pg/mL.

LLOQ = back interpolation of standard that provides CV≤ 20% and recovery ± 20% of the expected.

Expected IL-6 pg/mL	n	Mean O.D.	SD	CV%	LLOQ pg/mL	Calculated Mean IL-6 pg/mL	SD	CV	Recovery%
0.00	2	0.06	0.00	0	1.17				
1.17	2	0.08	0.00	0.9		1.13	0.03	2.7	97
2.34	2	0.11	0.00	1.9		2.30	0.09	4	98
4.69	2	0.17	0.01	3.4		4.79	0.24	5.1	102
9.38	2	0.28	0.00	0.5		9.51	0.06	0.6	101
18.75	2	0.48	0.00	0.7		18.61	0.16	0.8	99
37.50	2	0.9	0.02	2.6		37.07	1.06	2.9	99
75.00	2	1.71	0.04	2.5		75.68	2.13	2.8	101
150.00	2	3.1	0.04	1.4		149.41	1.9	1.3	100

4. Reagent Characterization & Confirmation

Each of the critical reagents within the assay was subjected to a range of physicochemical tests to examine the consistency of the raw material from lot to lot. Each test was subsequently used as a confirmatory test prior to their inclusion in the full manufacturing process.

Critical Reagents are regarded as

- Calibrator material (recombinant protein used in Standards & Quality Controls)
- Capture monoclonal antibody
- Detection monoclonal antibody (Pre and Post Biotinylation)

The following lots of the critical reagents were tested

Lot	Calibrator	Capture antibody	Detection antibody
1	028M4878V	RB1811028	RB1811029
2	015M4836V	RB1812003	RB1812002
3	NA	RB1912001	RB1912002

Following characterization assays were done

Characterization Assay	Information provided
1D Gel analysis	Percent Purity
Amino Acid Analysis (AAA)	Concentration in ug/mL of calibrator
Peptide Mapping (LC-MS)	Identification, sequence coverage and verification, Impurity estimation
RP-LC-UV-MS	Molecular weight, glycosylation profile
Biacore (SPR)	Binding kinetics and affinity

Detailed Test Results

a. 1D Gel analysis

This technique demonstrated that over all reagent purity was >98% (determined by a summary of 1D gel analysis for all reduced samples. Estimated molecular weight provided by comparison with a historical molecular weight marker).

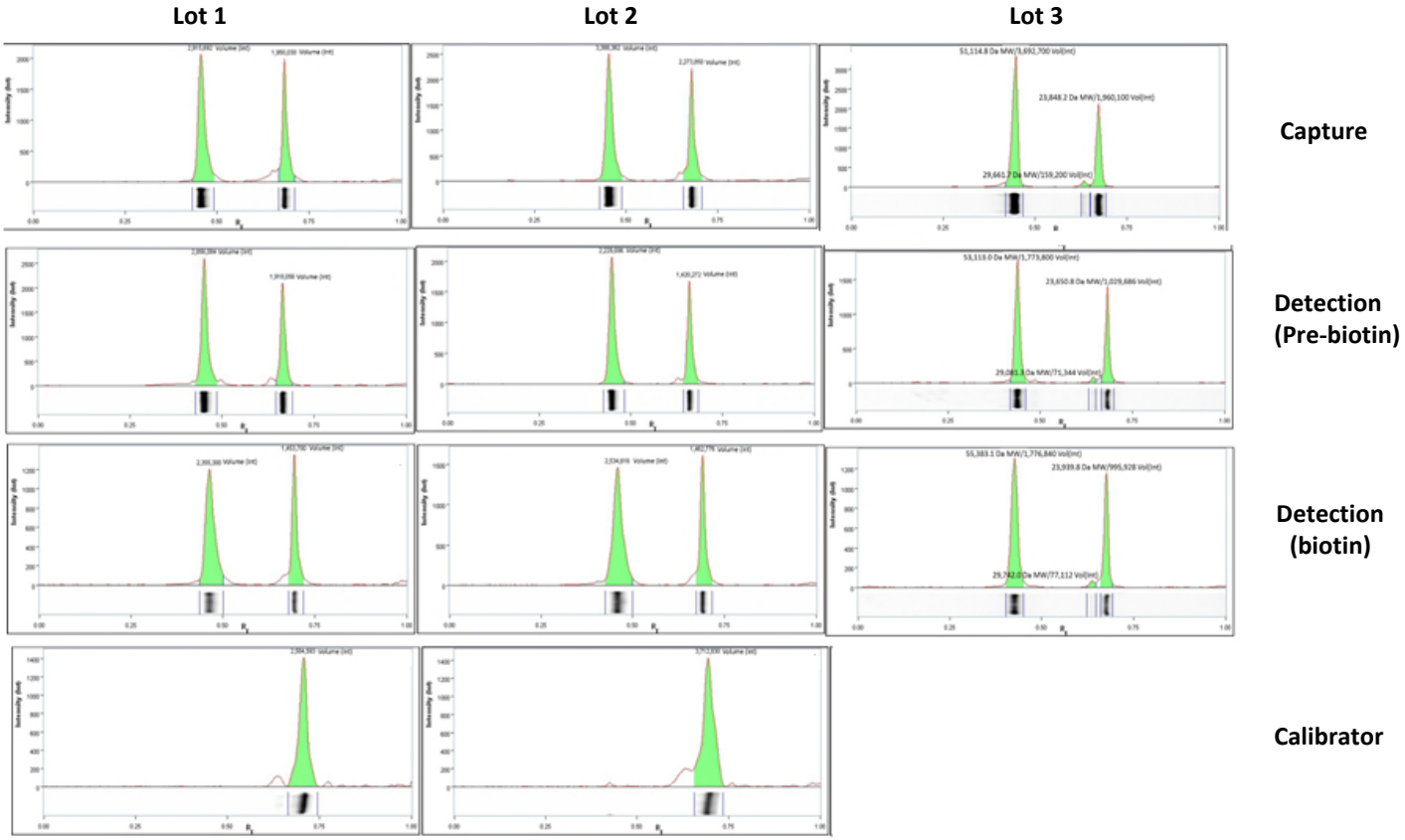

Figure 1: Densitograms of reduced samples by lot. From top to bottom, Capture mAb, Detection mAb, Detection mAb post biotinylation, and Calibrator material. Left to Right lot 1 -3 for each reagent where applicable.

b. AAA determination of Calibrator

To confirm the materials concentration, the calibrator was tested using Amino Acid Analysis with a NIST calibrator (SRM 2389) and a NIST BSA standard (SRM 927) for control purposes.

		All AAA (µg/mL)			vial content (µg/mL)		
No	**Calibrator**	**Rep 1**	**Rep 2**	**Rep 3**	**Mean**	**SD**	**%RSD**
1	Lot# 028M4878V	140.751	134.053	140.384	**138.40**	3.77	2.7
2	Lot# 015M4836V	154.477	176.465	183.233	**171.39**	15.03	8.8

Table 1: Amino Acid Analysis of two lots of IL-6 calibrator material against a NIST amino acid standard SRM 2389 using NIST BSA standard SRM 927 as an assay control

c. Calibrator sequence coverage analysis

Each of the two calibrator lots were examined for sequence coverage using LC-MS. Corrected sequence coverage using the mature sequence of IL6 was 81.9% for 028M4878V and 88.5% for 015M4836V. The intact mass analysis agrees 100% with the sequence which supports that the sequence matches with reported sequence from UniProt accession # P05231, AA30-212.

Sequence coverage for Calibrator Lot 028M4878V

VPPGEDSKDVAAPHRQPLTSSERIDKQIRYILDGISALRKETCNKSNMCESSKEALAENNLNLPKMAEKDGCFQSGFNEE
TCLVKIITGLLEFEVYLEYLQNRFESSEEQARAVQMSTKVLIQFLQKKAKNLDAITTPDPTTNASLLTKLQAQNQWLQDM
TTHLILRSFKEFLQSSLRALRQM

Sequence coverage for Calibrator Lot 015M4836V

VPPGEDSKDVAAPHRQPLTSSERIDKQIRYILDGISALRKETCNKSNMCESSKEALAENNLNLPKMAEKDGCFQSGFNEE
TCLVKIITGLLEFEVYLEYLQNRFESSEEQARAVQMSTKVLIQFLQKKAKNLDAITTPDPTTNASLLTKLQAQNQWLQDM
TTHLILRSFKEFLQSSLRALRQM

Figure 2: Sequence coverage of IL-6 calibrator. The yellow highlight shows the identified sequences. The sequence coverage for 015M4836V and 028M4878V was obtained to be 81.9 and 88.5%, respectively.

d. RP-LC-UV-MS

Samples were tested to ensure similar measured mass was achieved between batches (+/- 0.2%), spectra comparison and an estimation of the biotin incorporation ratio was provided. A summary of the data for each lot of reagent is provided in Table 2 below.

Sample	Measured masses (Da)	Moles of Biotin/moles of antibody
Lot 028M4878V	20977	NA
Lot 015M4836V	20977	NA
IL6.2F2 (Lot RB1811028)	148728	NA
IL6.2F2 (Lot RB1812003)	148735	NA
IL6.2F2 (Lot RB1912001)	148728	NA
IL6.2E3 (Lot RB1811029)	148937	NA
IL6.2E3 (Lot RB1812002)	148934	NA
IL6.2E3 (Lot RB1912002)	148933	NA

IL6.2E3 Bt (Lot RB1811029)	Distribution with Bt=4 at 150744	3
IL6.2E3 Bt (Lot RB1812002)	Distribution with Bt=4 at 150744	4
IL6.2E3 Bt (Lot RB1912002)	Distribution with Bt=4 at 150748	8

Table 2: Summary of the RP-LC-UV-MS data; measured mass in Dalton (Da) and an estimation of the available biotin per mole of detection antibody. The RP-LC-UV-MS initially confirmed that each batch of the mAbbs and calibrator was of a similar size. The number of biotins per molecule of the detection Mab ranged from 3 to 8 for the three lots of detection mAbs. The effect of this slight variation was evaluated during the manufacture and QC of each subsequent lot of the assay.

e. Biacore Analysis

The binding affinity between the calibrator and the capture or detection antibodies (pre/post biotinylation), the K_D (dissociation constant) and % activity was determined using a Biacore T200 platform. Typically, a K_D value of <2nM indicates very high affinity between an antibody and the calibrator.

	Ligand (Antibody)	IL-6 Calibrator	K_D (nM)	% Activity
1	17M4830V-control	028M4878V	0.04	85
2	IL6.2F2 Capture RB1811028	015M4836V	0.13	86
3	IL6.2F2 Capture RB1811028	028M4878V	0.05	84
4	IL6. 2F2 Capture RB1812003	015M4836V	0.14	88
5	IL6. 2F2 Capture RB1812003	028M4878V	0.06	88
6	IL6.2F2 Capture RB1912001	028M4878V	0.05	93
7	IL6.2E3 Detection RB1811029	015M4836V	0.13	67
8	IL6.2E3 Biotinylated Detection RB1811029	015M4836V	0.11	63
9	IL6.2E3 Detection RB1811029	028M4878V	0.08	70
10	IL6.2E3 Biotinylated Detection RB1811029	028M4878V	0.06	68
11	IL6.2E3 Detection RB1812002	015M4836V	0.11	66
12	IL6.2E3 Biotinylated Detection RB1812002	015M4836V	0.12	64
13	IL6.2E3 Detection RB1812002	028M4878V	0.10	72
14	IL6.2E3 Biotinylated Detection RB1812002	028M4878V	0.10	68
15	IL6.2E3 Detection RB1912002	028M4878V	0.06	83
16	IL6.2E3 Biotinylated Detection RB1912002	028M4878V	0.06	75

Table 3: Affinity and activity of three lots of Capture and Detection mAb (pre & post biotinylation) against the two calibrator lots

Each lot of the capture mAb performed similarly in terms of activity, while demonstrating a strong affinity for the calibrator (<0.2nM). The pre-biotinylated detection mAb also demonstrated a strong affinity for the calibrator. The biotinylation of the detection mAb did not significantly affect binding affinity.

Independent kit performance confirmatory study

Three lots of the IL-6 ELISA kits were produced using the following reagents and sent to Washington University, St Louis, Core laboratory for analysis.

	Lot 1	Lot 2	Lot 3
Capture Mab	RB1811028	RB1812003	RB1912001
Detection Mab	RB1811029	RB1812002	RB1912002
Calibrator Material	028M4878V	028M4878V	028M4878V

In the hands of a single user, three lots of calibration curves were compared to assess lot to lot variability

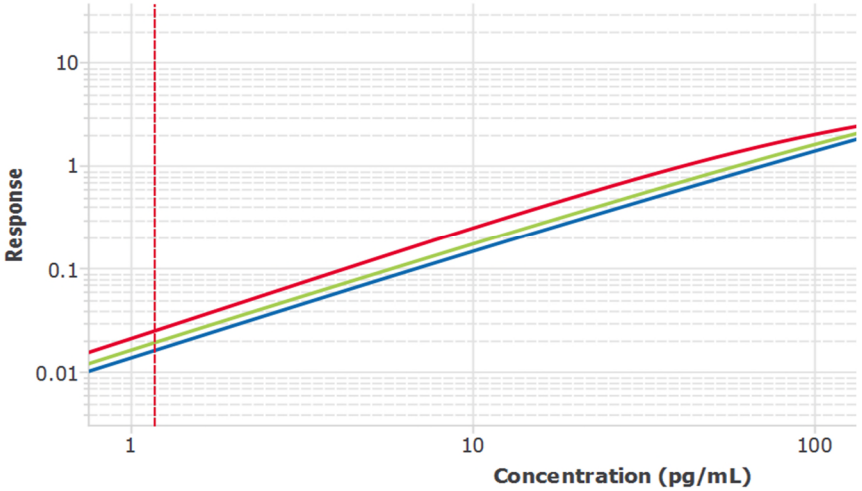

Fig 3: Mathematical parallelism among three standard curves from three lots of IL-6 ELISA using Belysa™ Immunoassay curve fitting software

Using the Belysa™ Immunoassay curve fitting software (Cat# 40-122) parallelism tool, Lot 1 was used as the reference curve, to which lot 2 and lot 3 were compared (Table 5). The three lots of IL-6 ELISA kits demonstrated excellent lot to lot reproducibility.

Curve	Parallelism
lot2 vs lot 1	1.035
lot 3 vs lot 1	1.001

Table 5: Belysa™ curve comparison results when using lot 1 as a reference against lot 2 & lot 3. Mathematical variation between curves is assed as perfect when parallelism is equal to 1.0 +/- 0.1.

Summary

The reagents used to create the initial lots of kits and the first production lot showed a high degree of purity and consistency. This was corroborated by the three-lot test by Washington University St. Louis where the curves demonstrated excellent mathematical correlation. As such the methods applied will continue to be used for ongoing analysis of the critical component raw materials to ensure that lot to lot kit reproducibility is maintained.

5. Spike Recovery

10 neat samples (5 serum and 5 K2 EDTA plasma) were spiked with four different concentrations of recombinant IL-6 protein, 0 pg/ml (assay buffer), 4.69 pg/ml, 9.38 pg/mL and 18.75 pg/mL to analyze recovery**.** Spike recovery is calculated as:

$$\frac{\textbf{interpolated spiked sample} - \textbf{interpolated endogenous}}{\textbf{Nominal Spike}} \times \mathbf{100}\%$$

Spike Recovery Results

Spike recovery ranged between 91% -107% with a mean of 97% for serum and between 96% -109% with a mean of 103% for plasma.

Serum Spike Recovery

Sample	n	Spiked IL-6 pg/mL	Sample Vol. (µL/well)	Mean IL-6 pg/mL	SD	CV%	% Spike Recovery
Serum 1	2	0	50	0	0	0	
	2	4.69		4.43	0.04	1.0	94
	2	9.38		8.98	0.15	1.7	96
	2	18.75		17.74	0.39	2.2	95
Serum 2	2	0	50	0	0	0	
	2	4.69		5.01	0.08	1.6	107
	2	9.38		9.88	0.19	1.9	105
	2	18.75		18.87	0.20	1.0	101
Serum 3	2	0	50	3.54	0.30	8.6	
	2	4.69		8.18	0.27	3.3	99
	2	9.38		12.63	0.16	1.2	97
	2	18.75		22.13	0.20	0.9	99
Serum 4	2	0	50	0.39	0	0	
	2	4.69		4.76	0.35	7.3	93
	2	9.38		9.22	0.11	1.2	94
	2	18.75		17.96	0.23	1.3	94
Serum 5	2	0	50	0.33	0	0	
	2	4.69		4.62	0.16	3.4	91
	2	9.38		8.89	0.27	3.0	91
	2	18.75		17.47	0.39	2.2	91

Plasma Spike Recovery

Sample	n	Spiked IL-6 pg/mL	Sample Vol. (µL/well)	Mean IL-6 pg/mL	SD	CV%	% Spike Recovery
Plasma 1	2	0	50	0.63	0	0	
	2	4.69		5.55	0.11	2.0	105
	2	9.38		10.04	0.22	2.2	100
	2	18.75		21.16	0.37	1.8	109
Plasma 2	2	0	50		0	0	
	2	4.69		4.53	0.15	3.3	96
	2	9.38		9.25	0.07	0.8	99
	2	18.75		19.71	0.86	4.3	105
Plasma 3	2	0	50	0.74	0	0	
	2	4.69		5.60	0.11	2.0	104
	2	9.38		10.07	0.04	0.4	99
	2	18.75		20.52	0.52	2.5	105
Plasma 4	2	0	50	0.45	0.04	7.9	
	2	4.69		5.34	0.11	2.1	104
	2	9.38		9.91	0.33	3.4	101
	2	18.75		19.21	0.15	0.8	100
Plasma 5	2	0	50	0.37	0	0	
	2	4.69		5.23	0.11	2.2	104
	2	9.38		10.14	0.23	2.2	104
	2	18.75		20.82	0.56	2.7	109

6. Parallelism/ Dilutional Linearity

Parallelism/ Dilutional Linearity Procedure

1. A dilution series was run on 10 normal and 10 diseased samples as follows
   a. 10 normal samples (5 serum and 5 plasma) were spiked with 20 pg/mL of IL-6 standard to measure dilution linearity.
   b. The samples were serially diluted 2-fold up to 1:8 dilution using serum matrix prior to running the assay.
   c. 10 samples with high endogenous levels of IL-6 (5 serum and 5 K2 EDTA samples) as determined by other studies were used to measure parallelism
   d. The samples were then serially diluted 2-fold up to 1:8 dilution using serum matrix prior to running the assay.

2. Dilution linearity/ parallelism is calculated as follows:

$$\frac{\textbf{Observed concentration}}{\textbf{Expected Concentration}} \times \textbf{100}\%$$

Where

1- Observed concentration is the mean calculated dilution-corrected concentration at each dilution
2- Expected concentration is the mean calculated dilution-corrected concentration of sample at the recommended dilution

Parallelism Results

Parallelism ranged between 83% -102% with a mean of 94% for serum samples and between 89% -110% with a mean of 100% for plasma samples

Sample Type	Condition	n	Dilution Condition	Mean IL-6 pg/mL	Dilution corrected IL-6 pg/mL	SD	CV%	% parallelism
Serum 1	Rheumatoid Arthritis	2	Neat	6.00	6.00	0.25	4.1	
		2	1:2	2.95	5.90	0.00	0.0	98
		2	1:4	1.37	5.48	0.23	4.1	91
		2	1:8					
Serum 2	Sepsis	2	Neat	52.20	52.20	1.05	2.0	
		2	1:2	26.25	52.50	0.93	1.8	101
		2	1:4	13.18	52.72	2.04	3.9	101
		2	1:8	6.34	50.72	1.92	3.8	97
Serum 3	Sepsis	2	Neat	31.31	31.31	1.14	3.6	
		2	1:2	14.87	29.74	0.71	2.4	95
		2	1:4	6.94	27.76	1.47	5.3	89
		2	1:8	3.31	26.44	1.19	4.5	84
Serum 4	Sepsis	2	Neat	28.71	28.71	1.34	4.7	
		2	1:2	14.58	29.15	0.89	3.1	102
		2	1:4	6.90	27.60	0.74	2.7	96
		2	1:8	3.31	26.44	1.64	6.2	92
Serum 5	Sepsis	2	Neat	55.46	55.46	0.33	0.6	
		2	1:2	26.94	53.87	1.48	2.8	97
		2	1:4	12.66	50.64	1.19	2.3	91
		2	1:8	5.73	45.84	0.00	0.0	83

Sample Type	Condition	n	Dilution Condition	Mean IL-6 pg/mL	Dilution corrected IL-6 pg/mL	SD	CV%	% parallelism
Plasma 1	Normal	2	Neat	16.51	16.51	0.04	0.2	
		2	1:2	8.40	16.80	0.06	0.3	102
		2	1:4	4.13	16.50	0.71	4.3	100
		2	1:8	1.97	15.76	0.00	0.0	95
Plasma 2	Normal	2	Neat	3.89	3.89	0.03	0.7	
		2	1:2	1.89	3.78	0.23	6.0	97
		2	1:4	0.88	3.52	0.34	9.6	90
		2	1:8					
Plasma 3	Rheumatoid Arthritis	2	Neat	36.23	36.23	1.22	3.4	
		2	1:2	19.28	38.56	2.60	6.7	106
		2	1:4	9.35	37.38	1.50	4.0	103
		2	1:8	4.52	36.16	2.15	5.9	100
Plasma 4	Rheumatoid Arthritis	2	Neat	59.32	59.32	1.87	3.1	
		2	1:2	29.48	58.96	2.88	4.9	99
		2	1:4	14.33	57.30	0.65	1.1	97
		2	1:8	6.62	52.92	2.21	4.2	89
Plasma 5	Rheumatoid Arthritis	2	Neat	10.50	10.50	0.37	3.6	
		2	1:2	5.78	11.56	0.59	5.1	110
		2	1:4	2.89	11.56	0.34	2.9	110
		2	1:8	1.25	10.00	0.00	0.0	95

Dilutional Linearity Results

The linearity ranged from 105%-116% with an overall average of 110% for serum samples. The linearity ranged from 88% - 105% with an overall average of 97% for plasma samples.

Serum Linearity

Sample Type	n	Dilution Condition	Mean IL-6 pg/mL	Dilution corrected IL-6 pg/mL	SD	CV%	% linearity
Serum 1	2	Neat	16.09	16.09	0	0	
	2	1:2	8.51	17.01	0.13	0.7	106
	2	1:4	4.45	17.78	0.14	0.8	111
	2	1:8	2.33	18.64	0.79	4.2	116
Serum 2	2	Neat	17.67	17.67	0.19	1.1	
	2	1:2	9.51	19.01	0.38	2.0	108
	2	1:4	4.91	19.64	0.11	0.6	111
	2	1:8	2.52	20.16	1.36	6.7	114

Serum 3	2	Neat	20.28	20.28	0	0	
	2	1:2	10.75	21.49	0.44	2.0	106
	2	1:4	5.67	22.68	0.79	3.5	112
	2	1:8	2.83	22.64	1.58	7.0	112
Serum 4	2	Neat	17.98	17.98	0.64	3.5	
	2	1:2	9.46	18.91	0.78	4.1	105
	2	1:4	4.94	19.74	0.25	1.3	110
	2	1:8	2.54	20.32	0.57	2.8	113
Serum 5	2	Neat	16.74	16.74	0.10	0.6	
	2	1:2	9.01	18.01	0.38	2.1	108
	2	1:4	4.70	18.80	0.28	1.5	112
	2	1:8	2.40	19.20	0.57	2.9	115

Plasma Linearity

Sample Type	n	Dilution Condition	Mean IL-6 pg/mL	Dilution corrected IL-6 pg/mL	SD	CV%	% Linearity
Plasma 1	2	Neat	20.01	20.01	0.15	0.7	
	2	1:2	9.97	19.93	1.60	8.0	100
	2	1:4	4.90	19.58	0.31	1.6	98
	2	1:8	2.21	17.68	0.57	3.2	88
Plasma 2	2	Neat	19.61	19.61	0.04	0.2	
	2	1:2	9.50	19.00	1.02	5.4	97
	2	1:4	4.54	18.16	1.13	6.2	93
	2	1:8	2.29	18.28	0.85	4.6	93
Plasma 3	2	Neat	19.77	19.77	0.11	0.5	
	2	1:2	9.84	19.67	0.07	0.4	100
	2	1:4	4.98	19.90	0.14	0.7	101
	2	1:8	2.34	18.68	1.98	10.6	95
Plasma 4	2	Neat	21.05	21.05	0.22	1.0	
	2	1:2	10.27	20.54	0.74	3.6	98
	2	1:4	5.18	20.70	1.27	6.1	98
	2	1:8	2.49	19.88	0.85	4.3	94
Plasma 5	2	Neat	19.65	19.65	0.18	0.9	
	2	1:2	9.80	19.60	0.68	3.5	100
	2	1:4	5.00	19.98	0.59	3.0	102
	2	1:8	2.58	20.64	1.81	8.8	105

7. Precision

Assay Precision Procedure

To assess the intra- and inter-assay precision of the IL-6 assay, five normal samples were run in duplicate on 3 days by 3 different operators.

Assay Precision Results

		Intra-assay			Inter-assay		
Sample	n	Mean IL-6 pg/mL	SD	CV%	Mean IL-6 pg/mL	SD	CV%
49	2	15.45	0.78	5.0	17.61	1.87	10.6
	2	18.62	0.24	1.3			
	2	18.67	0.34	1.8			
54	2	10.10	0.71	7.0	10.67	0.51	4.7
	2	10.84	0.35	3.2			
	2	11.07	0.10	0.9			
61	2	3.1	0.00	0.0	2.87	0.36	12.5
	2	3.06	0.08	2.5			
	2	2.46	0.36	14.7			
59	2	10.70	1.13	10.6	10.60	0.61	5.8
	2	11.16	0.24	2.2			
	2	9.95	0.69	7.0			
65	2	6.25	0.49	7.9	6.20	0.24	3.9
	2	5.94	0.00	0.0			
	2	6.42	0.05	0.8			

8. Results for Serum & Plasma

- This assay has been verified in human serum and plasma (K2 EDTA, K3 EDTA, Sodium heparin, sodium citrate).
- No other matrices have been tested.

9. Results for Known Diseased Samples

- Serum and plasma samples from a few disease conditions like Rheumatoid Arthritis and sepsis were tested. IL-6 levels were found to be elevated in some of the samples.

10. Analyte Cross-Reactivity Testing

- This assay was tested for cross reactivity with mouse IL-6 and other human cytokines like IL-1a, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-12, IL-13, IL-15, TNFα, TNFβ, GMCSF, GCSF
- There is no cross-reactivity detected with either analyte.

11. Assay Format

- Capture: Monoclonal
- Detection: Monoclonal
- Analyte: Recombinant

12. WHO standard Evaluation

The Conferma™ Human IL-6 ELISA kit standard was evaluated against the WHO Reference Standard (NIBSC code 89/548). Following equation can be used to convert IL6 values in pg/ml to the corresponding WHO units.

IL6 sample values in IU/mL= 0.2 X concentration in pg/ml calculated from Conferma IL-6 kit