Human IL-6 High Sensitivity ELISA

For in vitro diagnostic use, not for therapeutic procedures

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950 035 096	**1 x 96 tests**
950 035 192	**2 x 96 tests**

INTENDED USE

The IL-6 High sensitivity ELISA is to be used for the in-vitro quantitative determination of interleukin-6 (IL-6) in human serum, plasma, buffered solutions or cell culture medium. The assay will recognize both natural and recombinant human IL-6 . **IL-6 H.S. ELISA is for in vitro diagnostic use. Not for therapeutic procedures.**

SUMMARY

Interleukin-6 (IL-6) is a multi-functional cytokine that regulates immune responses, acute phase reactions and hematopoiesis and may play a central role in host defense mechanisms (13, 31). The gene for human IL-6 has been localized to chromosome 7p21 (1). The genomic sequence has been determined (36). IL-6 is usually not produced constitutively by normal cells, but its expression is readily induced by a variety of cytokines (28), lipopolysaccharide (25) or viral infections (3). The IL-6 gene product is a single chain protein with a molecular mass ranging from 21 to 28 kDa, depending on the cellular source. Extensive posttranslational modifications like N- and O-linked glycosylation (20) as well as phosphorylation (21) seem to account for this heterogeneity. The cDNA fo IL-6 predicts a precursor protein of 212 amino acids (10). IL-6 is a pleiotropic cytokine produced by a variety of cells. It acts on a wide range of tissues, exerting growth-induction, growth-inhibition, and differentiation respectively, depending on the nature of the target cells.
IL-6 is involved in

- the induction of B-cell differentiation,
- the induction of acute phase proteins in liver cells,
- growth promotion of myeloma/plasmacytoma/hybridoma cells,
- induction of IL-2 and IL-2 receptor expression,
- proliferation and differentiation of T cells,
- inhibition of cell growth of certain myeloid leukemic cell lines and induction of their differentiation to macrophages,
- enhancement of IL-3-induced multipotential colony cell formation in hematopoietic stem cells and induction of maturation of megakaryocytes as a thrombopoietic factor,
- induction of mesangial cell growth,
- induction of neural differentiation of PC 12 cells and
- induction of keratinocyte growth (14).

The abnormal production of IL-6 was first suggested to be related to polyclonal B-cell activation with autoantibody production in patients with cardiac myxoma (9). Since then, IL-6 has been suggested to be involved in the pathogenesis of a variety of diseases. Measurement of I levels in serum and other body fluids thus provides more detailed insights into various pathological situations.

Infections:

Body fluids of patients with acute local bacterial or viral infections and serum of patients with gram-negative or positive bacteremia contain elevated levels of biologically active IL-6 (7, 16).

Obstetric Infections:
IL-6 has emerged as a reporter cytokine for intraamniotic infection (29).

Diseases associated with an altered immune system (polyclonal B-cell abnormalities or autoimmune diseases):
Elevated levels of circulating IL-6 have been detected in patients with cardiac myxoma (11), Castleman's disease (18), rheumatoid arthritis (12), IgM gammopathy and in those with acquired immunodeficiency syndrome (19, 23) as well as alcoholic liver cirrhosis (2, 32).

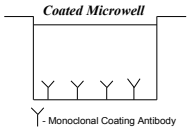
Proliferative diseases:
Elevated plasma levels of IL-6 are observed in patients with psoriasis (4, 5) and mesangial proliferative glomerulonephritis (15).

Neoplastic Diseases:
Increased systemic levels of IL-6 have been detected in patients with multiple myeloma (22), other B-cell dyscrasias (27), Lennert's T lymphoma, Castleman's disease, renal cell carcinoma (33) and various other solid tumors (17, 30).

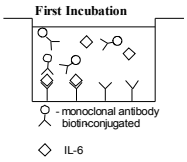
Inflammatory responses:
IL-6 is involved in the induction of acute phase proteins and induction of fever (8). Elevated serum levels of IL-6 are also found in patients with severe burns (24, 34), in serum and plasma as a marker for predicting postoperative complications (26), in serum and urine of recipients of kidney transplants before rejection (35), in the serum of septic shock patients (6) and in patients with inflammatory arthritis and traumatic arthritis.

PRINCIPLE OF THE TEST

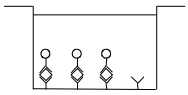
A monoclonal antibody specific for IL-6 has been coated onto the wells of the microtiter strips provided.



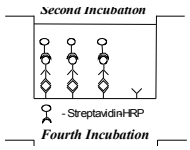
During the first incubation, IL-6 present in the sample or standard and a monoclonal anti IL-6 antibody conjugated to biotin are simultaneaously incubated.



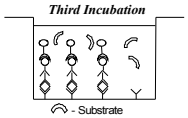
Following incubation unbound biotinylated anti-IL-6 is removed during a wash step.



Streptavidin-HRP is added and binds to the biotinylated anti IL-6. After incubation and a wash step a substrate solution reactive with HRP is added to the wells.



A coloured product is formed in proportion to the amount of IL-6 present in the sample. The reaction is terminated by addition of acid and absorbance is measured at 450 nm.



REAGENTS PROVIDED

REAGENTS (store at 2-8°C)	COLOUR CODE	QUANTITY 1X96 Tests 950 035 096	QUANTITY 2X96 Tests 950 035 192	RECONSTITUTION
96-wells precoated microtiter plate		1	2	Ready-to-use
Plate covers		2	4	
IL-6 Standard: 50 pg/ml	Yellow	2 vials	4 vials	Reconstitute with the volume of standard diluent indicated on the vial (see reagent preparation on page 5
Control	Silver	2 vials	4 vials	Reconstitute with the volume of standard diluent indicated on the vial (see reagent preparation on page 5
Standard Diluent Buffer	Black	1 bottle	1 bottle	(25 ml) 10X concentrate. Dilute in distilled water
Standard Diluent : human serum	Black	1 bottle	2 bottles	(7 ml) ready to use
Biotinylated anti IL-6	Red	1 vial	2 vials	(0.4 ml) Dilute in biotinylated antibody diluent
Biotinylated Antibody Diluent	Red	1 bottle (7.5ml)	1 bottle (13 ml)	Ready-to-use
Streptavidin-HRP		2 vials	4 vials	(5 µl) Add 0.5 ml of HRP-Diluent before further dilutions
HRP Diluent	Red	1 bottle	1 bottle	(23 ml) Ready to use
Washing Buffer	White	1 bottle	2 bottles	(10 ml) 200X concentrate. Dilute in distilled water
Substrate Solution ; chromogen TMB		1 bottle (11 ml)	1 bottle (24 ml)	Ready-to-use
Stop Reagent H_2SO_4	Black	1 bottle	2 bottles	(11 ml) Ready-to-use

MATERIALS REQUIRED BUT NOT PROVIDED

- 5 ml and 10 ml graduated pipettes
- 5 µl to 1,000 µl adjustable single channel micropipettes with disposable tips
- 50 µl to 300 µl adjustable multichannel micropipette with disposable tips
- Multichannel micropipette reservoir
- Vortex and Mixer
- Beakers, flasks, cylinders necessary for preparation of reagents
- Device for delivery of wash solution (multichannel wash bottle or automatic wash system)
- Microwell strip reader capable of reading at 450 nm (620 nm as optional reference wave length)
- Glass-distilled or deionized water
- Statistical calculator with program to perform linear regression analysis

SAFETY

- Reagents are intended for in vitro use and are not for use in therapeutic procedures.
- All chemicals in this kit should be considered as potentially hazardous. We therefore recommend that this product is handled only by persons who have been trained in laboratory techniques and that it is used in accordance with the principles of good laboratory practice.
- The human blood included in this kit have been tested and found non reactive for HbsAg, anti HIV1 & 2 and anti VHC. Nevertheless, no known method can offer complete assurance that human blood derivatives will not transmitted hepatitis, AIDS or other infections. Therefore handling of reagents, serum or plasma specimens should be in accordance with local safety procedures.
- Do not mix or substitute reagents with those from other lots or other sources.
- Do not use kit reagents beyond expiration date on label.
- Do not expose kit reagents to strong light during storage or incubation.
- Do not pipette by mouth.
- Do not eat or smoke in areas where kit reagents or samples are handled.
- Avoid contact of skin or mucous membranes with kit reagents or specimens.
- Rubber or disposable latex gloves should be worn while handling kit reagents or specimens.
- Thoroughly mix the reagents and samples before use by agitation or swirling.
- Reagents containing preservative may be toxic if ingested.
- Avoid contact of substrate solutions with oxidizing agents and metal.

- When pipetting reagents, maintain a consistent order of addition from well-to-well. This will ensure equal incubation times for all wells.
- Respect incubation times described in the assay procedure.
- Avoid splashing or generation of aerosols.
- In order to avoid microbial contamination or cross-contamination of reagents or specimens which may invalidate the test use disposable pipette tips and/or pipettes.
- Use a clean plastic container to prepare the washing solution.
- All residual washing liquid must be drained from the wells by efficient aspiration or by decantation followed by tapping the plate forcefully on absorbent paper. Never invert the absorbent paper directly into the wells.
- Use clean, dedicated reagent trays for dispensing the conjugate and substrate reagents.
- Exposure to acids will inactivate the conjugate.
- Glass-distilled water or deionized water must be used for reagent preparation.
- Substrate solutions must be at room temperature prior to use.
- Decontaminate and dispose specimens and all potentially contaminated materials as if they could contain infectious agents.The preferred method of decontamination is autoclaving for a minimum of 1 hour at 121.5°C.

STORAGE INSTRUCTIONS

Store kit reagents between 2° and 8°C. Immediately after use remaining reagents should be returned to cold storage (2° to 8°C). Expiry of the kit and reagents is stated on box front labels.
The expiry of the kit components can only be guaranteed if the components are stored properly, and if, in case of repeated use of one component, the reagent is not contaminated by the first handling.

SPECIMEN COLLECTION, PROCESSING AND STORAGE

Cell culture supernatants, human serum, plasma or other biological samples will be suitable for use in the assay. Remove serum from the clot or red cells, respectively, as soon as possible after clotting and separation.

Cell culture supernatants: Remove particulates and aggregates by spinning at approximately 1000 x g for 10 min.
Serum: Avoid any inintentional stimulation of the cells by the procedure. Use pyrogen/endotoxin free collecting tubes. Serum should be removed rapidly and carefully from the red cells after clotting. For that, after clotting, centrifuge at approximately 1000 x g for 10 min and remove serum.
Plasma: EDTA, citrate and heparin plasma can be assayed. Spin samples at 1000 x g for 30 min to remove particulates. Harvest plasma.
Storage: If not analyzed shortly after collection, samples should be aliquoted (250-500µl) to avoid freeze-thaw cycles and stored frozen at –70°C. Avoid multiple freeze-thaw cycles of frozen specimens.

Recommendation: Do not thaw by heating at 37°C or 56°C. Thaw at room temperature and make sure that sample is completely thawed and homogeneous before assaying

Samples containing a visible precipitate must be clarified prior to use in the assay. Do not use grossly hemolyzed or lipemic specimens.

For sample stability, please refer to Performance Characteristics, Sample stability.

PREPARATION OF REAGENTS

1. Washing Buffer

Dilute the **Washing Buffer Concentrate** (200X) in a clean graduated cylinder. Mix gently to avoid foaming. Pour entire contents (10 ml) of the Washing Buffer Concentrate into a clean 2,000 ml graduated cylinder. Bring final volume to 2,000 ml with glass-distilled or deionized water. Mix gently to avoid foaming
Transfer to a clean wash bottle and store at 2° to 25°C. Washing Buffer may be prepared as needed according to the following table:

Number of Strips	Washing Buffer Concentrate (ml)	Distilled Water (ml)
1 - 6	5	995
1 - 12	10	1,990

2. Preparation of Standard Diluent Buffer

Add the content of the vial (10X) to 225 ml distilled water before use.

3. Preparation of IL-6 Standards

Depending on the type of samples you are assaying, the kit includes two standard diluents. Because biologicals fluids might contain proteases or cytokine-binding proteins that could modify the recognition of the cytokine you want to measure. You should reconstitute standard vials with the most appropriate Standard Diluent.
For serum and plasma samples: use Standard Diluent : human serum
For cells culture supernatants: use Standard Diluent Buffer

Reconstitute IL-6 Standard by addition of appropriate diluent. Reconstitute volume is stated on the label of the standard vial.
This reconstitution produces a stock solution of 50 pg/ml IL-6 . Serial dilutions of standard must be made before each assays and cannot be stored.

4. Preparation of Controls

Freeze-dried control vials should also be reconstituted with the most appropriate Standard Diluent to your samples.
For serum and plasma samples: use Standard Diluent : human serum
For cells culture supernatants: use Standard Diluent Buffer.

Control have to be reconstituted with the volume indicated on the vial. Reconstitution of the freeze-dried material with the indicated volume, will give a solution for which the IL-6 concentration is stated on the vial. Do not store after use.

5. Preparation of biotinylated anti IL-6 .

Preparation immediately before use is recommended. Dilute the biotinylated anti-IL-6 with the biotinylated antibody diluent in a clean glass vial. Biotinylated anti IL-6 may be prepared as needed according the following table. Extemporaneous preparations are recommended.

Number of Strips	Biotinylated Antibody Concentrate (µl)	Biotinylated Antibody Diluent (µl)
2	40	1060
3	60	1590
4	80	2120
6	120	3180
12	240	6360

6. Preparation of Streptavidin-HRP

Dilute the Streptavidin-HRP 1:100 just prior to use by adding 0.5 ml of HRP diluent to the vial containing Streptavidin-HRP concentrate. DO NOT KEEP THIS DILUTION FOR FURTHER EXPERIMENTS.

Make a further dilution with HRP-Diluent in a clean glass vial as needed according to the following table:

Number of Strips	Pre-diluted Streptavidin-HRP (µl)	HRP Diluent (ml)
2	30	2
3	45	3
4	60	4
6	75	5
12	150	10

TEST PROTOCOL

a. Mix all reagents thoroughly without foaming before use.

b. Determine the number of microwell strips required to test the desired number of samples plus appropriate number of wells needed for running blanks and standards. Each sample, standard, blank, and control samples should be assayed in duplicate. Remove sufficient **Microwell Strips coated with Antibody to human IL-6** from the aluminium pouch immediately prior to use. Return any wells not required for this assay with desiccant to the pouch. Seal tightly.

c. Add 100µl of of **appropriate Standard Diluent** (see preparation of reagents) to standard wells B1, B2, C1, C2, D1, D2, E1, E2, F1, F2.
Reconstitute **standard vial** with the appropriate volume as described in the chapter preparation of reagents. Pipette 200 µl of standard into wells A1 and A2 (see Figure 1 and 2). Transfer 100 µl from A1 and A2 to B1 and B2 wells. Mix the contents by repeated aspirations and ejections. Take care not to scratch the inner surface of microwells. Repeat this procedure from the wells B1, B2 to wells C1, C2 and from wells C1, C2 to D1, D2 and so on creating two parallel rows of IL-6 standard dilutions ranging from 50 to 1.56 pg/ml. Discard 100 µl from the content of the last microwells used (F1, F2).
Alternatively these dilutions can be done in separate tube and diluted standard pipetted directly into wells.

Figure 1. Preparation of IL-6 standard dilutions:

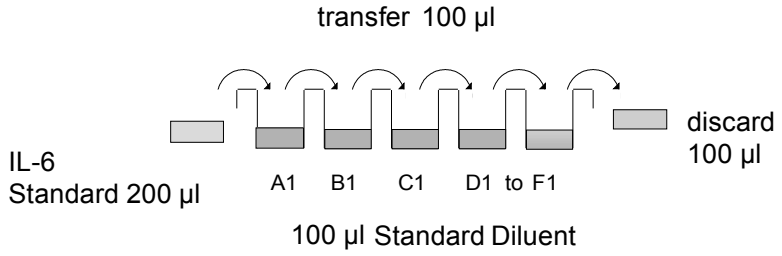


Figure 2. Diagram depicting an example of the arrangement of blanks, standards, samples and controls in the microwell strips:

	Standard Concentrations pg/mL		Sample wells									
	1	2	3	4	5	6	7	8	9	10	11	12
A	50	50										
B	25	25										
C	12.5	12.5										
D	6.25	6.25										
E	3.12	3.12										
F	1.56	1.56										
G	Blank	Blank										
H	Ctrl	Ctrl										

d. Add 100 µl of **appropriate Standard Diluent** in duplicate, to the blank wells (G1, G2).

e. Add 100 µl of **Sample** to sample wells, in duplicate, to the designated wells and 100 µl of **reconstituted control vial,** in duplicate, to control wells (H1, H2).

f. Prepare biotinylated anti IL-6 (refer to Preparation of reagents 5.).

g. Add 50 µl of **diluted biotinylated anti IL-6** to all wells.

h. Cover with a Plate Cover and incubate at room temperature (18° to 25°C) for 3 hours.

i. Remove the cover and wash the plate as follows:
   1. Aspirate the liquid from each well;
   2. Dispense 0.3 ml of washing solution into each well;
   3. Aspirate again the content of each well;
   4. Repeat step 2. and 3. two times

j. Prepare Streptavidin-HRP solution just before use: (refer to Preparation of reagents 6.)

k. Distribute 100 µl of **Streptavidin-HRP** solution to all wells, including blanks.

l. Cover the plate and incubate the plate at room temperature (18°C to 25°C) for 30 min.

m. Remove the cover and empty wells. Wash microwell strips according to step i. Proceed immediately to the next step.

n. Pipette 100 µl of ready-to-use **TMB Substrate Solution** to all wells, including the blank wells and incubate in the dark for 12-15 minutes at room temperature. Avoid direct exposure to light by wrapping the plate in aluminium foil.

o. Incubation time of the substrate solution is usually determined by the ELISA reader performances. Many ELISA readers record absorbance only up to 2.0 O.D. **Therefore the colour development within individual microwells must be watched by the person running the assay, and the substrate reaction stopped before positive wells are no longer properly recordable.**

p. The enzyme-substrate reaction is stopped by quickly pipetting 100 µl of **H_2SO_4 : Stop Reagent** into each well, including the blank wells to completely and uniformly inactivate the enzyme. Results must be read immediately after the H_2SO_4 : Stop Reagent is added.

q. Read absorbance of each microwell on a spectro-photometer using 450 nm as the primary wave length (optionally 620 nm as the reference wave length; 610 nm to 650 nm is acceptable). Blank the plate reader according to the manufacturer's instructions by using the blank wells. Determine the absorbance of both, the samples, controls and the IL-6 standards.

CALCULATION OF RESULTS

- Calculate the average absorbance values for each set of duplicate standards, samples and controls. Duplicates should be within 20 per cent of the mean.

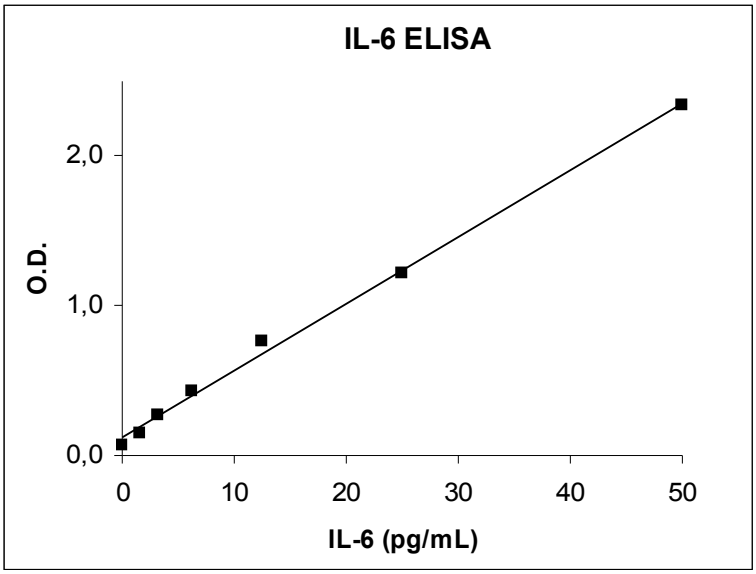
- Create a linear standard curve by plotting the mean absorbance for each standard concentration on the ordinate against the IL-6 standard concentration on the abscissa.

- To determine the concentration of IL-6 in each sample, first find the mean OD value on the ordinate and extend a horizontal line to the standard curve. At the point of intersection, extend a vertical line to the abscissa and read the corresponding IL-6 concentration.

  Note: Do not extrapolate the standard curve beyond the 50 pg/ml standard curve point. The dose-response is non-linear in this region and good accuracy is difficult to obtain. Concentrated samples (> 50 pg/ml) have to be diluted with standard diluent or with your own sample buffer. During analysis, multiply results by the appropriate dilution factor.

- A representative standard curve is shown in Figure 3. This curve cannot be used to derive test results. Every laboratory must prepare a standard curve for each group of microwell strips assayed.

Figure 3. Representative standard curve for IL-6 ELISA ranging from 1.56 to 50 pg/ml.
Do not use this standard curve to derive test results. A standard curve must be run for each group of microwell strips assayed.



Typical data using the IL-6 H.S. ELISA

Measuring wavelength: 450 nm
Reference wavelength: 620 nm

Standard	IL-6 Concentration (pg/ml)	O.D. (450 nm)	O.D. Mean	C.V. (%)
1	50 50	2.056 2.296	2.176	7.8
2	25 25	1.047 1.011	1.029	2.45
3	12.5 12.5	0.574 0.569	0.571	0.64
4	6.25 6.25	0.331 0.328	0.330	0.78
5	3.12 3.12	0.215 0.212	0.213	1.05
6	1.56 1.56	0.146 0.154	0.150	3.94
Blank	0 0	0.069 0.069	0.069	0.02

LIMITATIONS OF THE PROCEDURE

- Since exact conditions may vary from assay to assay, a standard curve must be established for every run.
- Bacterial or fungal contamination of either samples or reagents or cross-contamination between reagents may cause erroneous results.
- Disposable pipette tips, flasks or glassware are preferred, reusable glassware must be washed and thoroughly rinsed of all detergents before use.
- Improper or insufficient washing at any stage of the procedure will result in either false positive or false negative results. Completely empty wells before dispensing fresh Washing Buffer, fill with Washing Buffer as indicated for each wash cycle and do not allow wells to sit uncovered or dry for extended periods.

PERFORMANCE CHARACTERISTICS

Sensitivity

The limit of detection for IL-6 , defined as the analyte concentration resulting in an absorption significantly higher than the absorption of the dilution medium (mean plus three standard deviations) was determined to be 0.81 pg/ml. The sensitivity was determined by evaluation of at least 40 blanks (80 replicates) in one session.

Reproductibility

a. Intra-assay

Reproductibility within the assay was evaluated in three independent experiments by two different technicians. Each assay was carried out with 6 replicates (3 duplicates) of 3 spiked human pooled serum samples and 2 supernatants containing different concentrations of IL-6. Data below show the mean IL-6 . concentration and the coefficient of variation for each sample. **The overall intra-assay coefficient of variation has been calculated to be 4.4%.**

Sessions	Samples	Assay 1 [IL-6 H] pg/ml	Assay 2 [IL-6] pg/ml	Assay 3 [IL-6] pg/ml	Mean	SD	CV
1	1	24.10	23.50	23.60	23.7	0.3	1.35
	2	10.70	10.40	10.80	10.6	0.2	1.96
	3	5.30	5.80	5.90	5.7	0.3	5.67
	4	44.90	36.20	39.40	40.2	4.4	10.96
	5	27.90	30.50	31.90	30.1	2.0	6.74
2	1	25.30	25.10	26.90	25.8	1.0	3.83
	2	12.8	12.90	11.90	12.5	0.6	4.39
	3	5.40	5.05	5.60	5.4	0.5	5..2
	4	40.92	41.71	40.70	41.1	0.5	1.29
	5	31.55	32.60	31.44	31.9	0.6	2.01
3	1	23.13	23.72	21.97	22.9	0.9	3.88
	2	10.50	10.42	11.09	10.7	0.4	3.43
	3	4.88	5.22	5.59	5.2	0.4	6.79
	4	40.6	39.69	37.93	39.2	1.1	2.9
	5	27.01	29.99	29.94	29	1.7	5.89

Example operator A

b. Inter-assay

Assay to assay reproductibility within one laboratory was evaluated in three independent experiments by two technicians. Each assay was carried out with 6 replicates of 3 spiked serum human pooled serum samples and 2 supernatants containing different concentrations of IL-6 . **The calculated overall coefficient of variation was 9.1%**

Technician	Session	Sample 1 [IL-6 H.S.] pg/ml	Sample2 [IL-6 H.S.] pg/ml	Sample 3 [IL-6 H.S.] pg/ml	Sample 4 [IL-6 H.S.] pg/ml	Sample 5 [IL-6 H.S.] pg/ml
A	1	24.1	10.7	5.30	44.90	27.80
		23.5	10.4	5.8	36.20	30.50
		23.6	10.8	5.9	39.40	31..90
	2	25.30	12.8	5.40	41.70	31.60
		25.10	12.9	5.04	40.70	32.60
		26.90	11.90	5.60	40.70	31.40
	3	23.13	10.50	4.99	40.06	27.01
		23.72	10.42	5.22	39.67	29.99
		21.97	11.09	5.59	37.93	29.95
B	1	34.46	13.60	7.47	41.98	30.94
		26.70	12.55	6.97	40.48	31.85
		19.69	11.69	6.04	41.97	31.68
	2	28.44	12.43	7.65	43.56	29.95
		27.01	13.03	7.10	42.63	31.94
		25.78	12.66	6.58	40.14	31.11
	3	25.62	11.61	6.98	44.64	31.39
		24.21	10.86	6.37	38.08	29.31
		24.19	11.78	6.40	37.60	29.45
Mean		25	12	6	41	31
SD		3	1	1	2	1
CV		12.2	8.7	13.7	5.9	4.9

Spike Recovery

The spike recovery was evaluated by spiking three concentrations of recombinant IL-6 into human serum samples (or human pooled serum). As shown below recoveries were determined in 2 independent experiments with 6 replicates each. The unspiked diluent was used as blank in these experiments. **Recoveries ranged from 107% to 123% with an overall mean recovery of 115%.**

IL-6 Spike (pg/ml)	Experiment	Calculated concentration IL-6	Recovery (%) IL-6
50	1	56	111
	2	53	107
25	1	30	118
	2	31	123
12.5	1	15	119
	2	14	108

Dilution Parallelism

Three serum samples and one human pooled serum with different levels of IL-6 were analysed at different serial two fold dilutions with 2 replicates each. In the table below the per cent recovery of expected values is listed. **Recoveries ranged from 80% to 123% with an overall mean recovery of 93 %**

		IL-6 concentration (pg/ml)		
Sample	Dilution	Expected Value	Observed Value	% Recovery of Exp. Value
1	neat	24.0	24.0	--
	1:2	12.0	10.8	90
	1:4	5.4	5.0	93
	1:8	2.5	2.0	80
2	neat	--	24.0	--
	1:2	12.0	10.5	88
	1:4	5.3	5.5	104
	1:8	2.8	2.5	89
3	neat	--	24.0	--
	1:2	12.0	11.0	92
	1:4	5.5	5.5	100
	1:8	2.8	2.0	72
4	neat	--	26.0	--
	1:2	13.0	10.5	123
	1:4	5.5	6.5	85
	1:8	3.25	3.3	101

Sample stability

a. Freeze-Thaw Stability

Aliquots of serum samples (spiked) were stored at -20°C and thawed up to 5 times and the IL-6 levels determined. As shown in the table below. there was no significant loss of IL-6 after 5 cycles of freezing and thawing.

N° of Freeze	IL-6 Sample 1		IL-6 Sample 2	
Thaw cycles	Concentration (pg/ml)	Recovery (%)	Concentration (pg/ml)	Recovery (%)
0	29	-	41	-
1	29	100	42	102
3	34	117	48	117
5	33	114	47	115

b. Storage stability

Aliquots of 2 samples (spiked) were stored at -20°C. 2-8°C. room temperature (RT) and at 37°C. and the IL-6 level determined after 24h. As shown in the table below. there was no significant loss of IL-6 immunoreactivity during storage under above conditions.

Storage	IL-6 Spike
Temperature	Recovery (%)
-20°C	-
2-8°C	102
RT	114
37°C	100

Specificity

The assay recognizes both natural and recombinant human IL-6 . To define the specificity of this ELISA several proteins were tested for cross reactivity. There was no cross reactivity observed for any protein tested (IL-1α, IL-1β, IL-10, IL-12, IFNγ, IL-4, TNFα, IL-8 and IL-13).

Expected serum values

A panel of 20 human serum had been performed. The detected IL-6 levels ranged between : below the detection level and 4.72pg/ml with a mean level at 1.3pg/ml and a standard deviation of +/-1.4pg/ml.

Standard Calibration

This immunoassay is calibrated against the International Reference Standard NIBSC 89/548. NIBSC 89/548 is quantitated in International Units (IU). 1IU corresponding to 11 pg Diaclone IL-6 H.S..

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REAGENT PREPARATION SUMMARY

1. Washing Buffer Add **Washing Buffer Concentrate** 200 X (10 ml) to 1990 ml distilled water

2 Standard Diluent Buffer Add **Standard Diluent Buffer Concentrate** 10 X (25 ml) to 225 ml distilled water

3. IL-6 Standard Reconstitute **IL-6 Standard** by addition of appropriate Standard Diluent as stated on vial label.

4. Controls Reconstitute **controls** by addition of appropriate Standard Diluent as stated on vial label.

5. Biotinylated anti IL-6

Number of Strips	Biotinylated Antibody Concentrate (µl)	Biotinylated Antibody Diluent (µl)
2	40	1060
3	60	1590
4	80	2120
6	120	3180
12	240	6360

6. Streptavidin-HRP

Number of Strips	Pre-Diluted Streptavidin-HRP (µl)	HRP-Diluent (ml)
2	30	2
3	45	3
4	60	4
6	75	5
12	150	10

TEST PROTOCOL SUMMARY: Total procedure length: 3h45mn

- Add 100 µl of appropriate Standard Diluent Buffer. in duplicate. to standard wells (B1 to F2)
- Pipette 200 µl reconstituted IL-6 Standard in wells A1 and A2 and create standard dilutions ranging from 50 to 1.56 pg/ml by transferring 100 µl from well to well. Discard 100 µl from the last wells.
- Add 100 µl of appropriate **Standard Diluent Buffer** . in duplicate. to the blank wells.
- Add 100 µl of **Sample**. in duplicate. to designated wells and 100 µl of reconstituted **control**. in duplicate. to control wells.
- Prepare Biotinylated anti IL-6 .
- Add 50 µl of **diluted biotinylated anti IL-6 .** to all wells
- Cover microwell strips and incubate 3 hours at room temperature (18-25°C)
- Empty and wash microwell strips 3 times with **Washing Buffer**
- Prepare Streptavidin-HRP
- Add 100 µl of **diluted Streptavidin-HRP** to all wells
- Incubate 30 minutes covered at room temperature (18° to 25°C).
- Empty and wash microwell strips 3 times with **Washing Buffer**
- Add 100 µl of ready-to-use **TMB solution** to all wells including blank wells.
- Incubate the microwell strips for about 12-15 minutes at room temperature (18° to 25°C) in the dark.
- Add 100 µl H_2SO_4: **Stop Solution** to all wells including blank wells.
- Measure colour intensity at 450 nm and optinally at 620 nm as the reference wave length (610 nm to 650 nm is acceptable).

Note: Calculation of samples with an O.D. exceeding the range of the standard curve may result in incorrect. low IL-6 levels. Such samples require further dilution with appropriate Standard Diluent Buffer in order to precisely quantitate the actual IL-6 level.