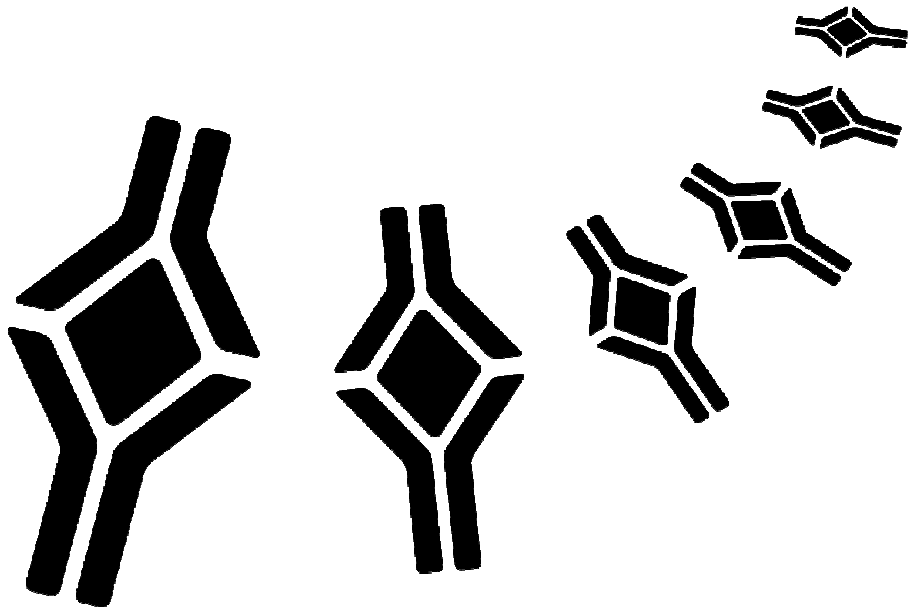

HUMAN INTERLEUKIN-6 ELISA, HIGH SENSITIVITY

Product Data Sheet

Cat. No.: RGP013R

For Research Use Only

VERSION 51 121012 46

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» **This kit is manufactured by:**
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» **Use only the current version of Product Data Sheet enclosed with the kit!**

1. INTENDED USE

The Human Interleukin-6 ELISA, High Sensitivity is a solid phase sandwich ELISA for research use only quantitative determination of IL-6 in supernatants, buffered solutions or serum and plasma samples and other body fluids. This assay will recognise both natural and recombinant human IL-6.

This kit has been configured for research only. Not suitable for use in therapeutic procedures.

2. INTRODUCTION

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 for 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 IL-6 levels in serum and other body fluids thus provides more detailed insights into various pathological situations. For Example:

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.

3. PRINCIPLE OF THE METHOD

A capture Antibody highly specific for IL-6 has been coated to the wells of the microtitre strip plate provided during manufacture. Binding of IL-6 samples and known standards to the capture antibodies and subsequent binding of the biotinylated anti-IL-6 secondary antibody to the analyte is completed during the same incubation period. Any excess unbound analyte and secondary antibody is removed. The HRP conjugate solution is then added to every well including the zero wells, following incubation excess conjugate is removed by careful washing. A chromogen substrate is added to the wells resulting in the progressive development of a blue coloured complex with the conjugate. The colour development is then stopped by the addition of acid turning the resultant final product yellow. The intensity of the produced coloured complex is directly proportional to the concentration of IL-6 present in the samples and standards.

The absorbance of the colour complex is then measured and the generated OD values for each standard are plotted against expected concentration forming a standard curve. This standard curve can then be used to accurately determine the concentration of IL-6 in any sample tested.

4. REAGENTS PROVIDED AND RECONSTITUTION

REAGENTS (Store at 2-8°C)	**Quantity**	**State**
Antibody Coated Microtiter Strips	96 wells	Ready to use (Pre-coated)
Plastic plate covers	2 vials	n/a
Standard: 50 pg/ml	2 vials	Reconstitute as directed in Quality Control Sheet (see reagent preparation)
Standard Diluent (Buffer)	1 vial (25 ml)	10x Concentrate, dilute in distilled water (see reagent preparation)
Standard Diluent (Human serum)	1 (7ml)	Ready to use
Control	2 vials	Reconstitute as directed in Quality Control Sheet (see reagent preparation)
Biotinylated anti-IL-6	1 vial (0.4 ml)	Dilute in biotinylated antibody diluent (see reagent preparation)
Biotinylated Antibody diluent	1 vial (7 ml)	Ready to use
Streptavidin-HRP	2 vials (5ml)	Add 0.5ml of HRP diluent prior to use (see reagent preparation)
HRP Diluent	1 vial (23 ml)	Ready to use
Wash Buffer	1 vial (10 ml)	200x Concentrate dilute in distilled water (see reagent preparation)
TMB Substrate:	1 vial (11 ml)	Ready to use
H_2SO_4 : Stop Reagent	1 vial (11 ml)	Ready to use

5. MATERIAL REQUIRED BUT NOT PROVIDED

- Microtitre plate reader fitted with appropriate filters (450 nm required with optional 620 nm reference filter)
- Microplate washer or wash bottle
- 10, 50, 100, 200 and 1,000 µl adjustable single channel micropipettes with disposable tips
- 50-300 ml multi-channel micropipette with disposable tips
- Multichannel micropipette reagent reservoirs
- Distilled water
- Vortex mixer
- Miscellaneous laboratory plastic and/or glass, if possible sterile

6. STORAGE INSTRUCTIONS

Store kit reagents between 2 and 8°C. Immediately a fter use remaining reagents should be returned to cold storage (2-8°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.

7. SPECIMEN COLLECTION, PROCESSING & 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: Use pyrogen/endotoxin free collecting tubes. Serum should be removed rapidly and carefully from the red cells after clotting. Following 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 (250-500 µl) to avoid repeated freeze-thaw cycles and stored frozen at –70°C. Avoi d multiple freeze-thaw cycles of frozen specimens.

Recommendation: Do not thaw by heating at 37°C or 56°C. Thaw at roo m temperature and make sure that sample is completely thawed and homogeneous before use. When possible avoid use of badly haemolysed or lipemic sera. If large amounts of particles are present these should be removed prior to use by centrifugation or filtration.

8. SAFETY & PRECAUTIONS FOR USE

- Handling of reagents, serum or plasma specimens should be in accordance with local safety procedures , e.g.CDC/NIH Health manual : " Biosafety in Microbiological and Biomedical Laboratories" 1984
- The human serum 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 transmit hepatitis, AIDS or other infections. Therefore handling of reagents, serum or plasma specimens should be in accordance with local safety procedures
- Laboratory gloves should be worn at all times
- Avoid any skin contact with H_2SO_4 and TMB. In case of contact, wash thoroughly with water
- Do not eat, drink, smoke or apply cosmetics where kit reagents are used
- Do not pipette by mouth
- When not in use, kit components should be stored refrigerated or frozen as indicated on vials or bottles labels
- All reagents should be warmed to room temperature before use. Lyophilized standards should be discarded after use
- Once the desired number of strips has been removed, immediately reseal the bag to protect the remaining strips from deterioration
- Cover or cap all reagents when not in use
- Do not mix or interchange reagents between different lots
- Do not use reagents beyond the expiration date of the kit
- Use a clean disposable plastic pipette tip for each reagent, standard, or specimen addition in order to avoid cross contamination, for the dispensing of H_2SO_4 and substrate solution, avoid pipettes with metal parts
- Use a clean plastic container to prepare the washing solution
- Thoroughly mix the reagents and samples before use by agitation or swirling
- 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 insert absorbent paper directly into the wells
- The TMB solution is light sensitive. Avoid prolonged exposure to light. Also, avoid contact of the TMB solution with metal to prevent colour development. Warning TMB is toxic avoid direct contact with hands. Dispose off properly
- If a dark blue colour develops within a few minutes after preparation, this indicates that the TMB solution has been contaminated and must be discarded. Read absorbance's within 1 hour after completion of the assay
- When pipetting reagents, maintain a consistent order of addition from well-to-well. This will ensure equal incubation times for all wells
- Follow incubation times described in the assay procedure
- Dispense the TMB solution within 15 min of the washing of the microtitre plate

9. ASSAY PREPARATION

Bring all reagents to room temperature before use

9.1 Assay Design

Determine the number of microwell strips required to test the desired number of samples plus appropriate number of wells needed for running zeros and standards. Each sample, standard, zero and control should be tested in duplicate. Remove sufficient Microwell Strips for testing from the aluminium pouch immediately prior to use. Return any wells not required for this assay with desiccant to the pouch. Seal tightly and return to 2-8°C storage.

Example plate layout (example shown for a 6 point standard curve)

	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	Zero	Zero										
H	CTRL	CTRL										

Standards / Controls — **Sample wells**

All remaining empty wells can be used to test samples in duplicate

9.2. Preparation of Wash Buffer

Dilute the (200x) wash buffer concentrate 200 fold with distilled water to give a 1x working solution. 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°-25°C.

9.3. Preparation of Standard Diluent Buffer

Add the contents of the vial (10x concentrate) to 225 ml of distilled water before use.
This Solution can be stored at 2-8°C for up to 1 week.

9.4. Preparation of Standard

Depending on the type of samples you are assaying, the kit may include two standard diluents. Because biological 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 **cell culture supernatants**: use Standard Diluent Buffer
Standard vials must be reconstituted with the volume of standard diluent shown in Quality Control Sheet immediately prior to use. This reconstitution gives a stock solution of 50 pg/ml of IL-6. Mix the reconstituted standard gently by repeated aspirations/ejections. Serial dilutions of the standard are made directly in the assay plate to provide the concentration range from 50 to 1.56 pg/ml. A fresh standard curve should be produced for each new assay.

- Immediately after reconstitution add 200 ml of the reconstituted standard to well's A1 and A2, which provides the highest concentration standard at 50 pg/ml
- Add 100 ml of appropriate standard diluent to the remaining standard wells B1 and B2 to F1 and F2
- Transfer 100 ml from wells A1 and A2 to B1 and B2. Mix the well contents by repeated aspirations and ejections taking care not to scratch the inner surface of the wells
- Continue this 1:1 dilution using 100 ml from wells B1 and B2 through to wells F1 and F2 providing a serial diluted standard curve ranging from 50 pg/ml to 1.56 pg/ml
- Discard 100 ml from the final wells of the standard curve (F1 and F2) Alternatively these dilutions can be performed in separate clean tubes and immediately transferred directly into the relevant wells.

9.5. 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
The supplied Controls must be reconstituted with the volume of Standard Diluent indicated in Quality Control Sheet. Reconstitution of the freeze-dried material with the indicated volume, will give a solution at the concentration stated on the vial. Do not store after use.

9.6. Preparation of Biotinylated anti-CD138

It is recommended this reagent is prepared immediately before use. Dilute the biotinylated anti-IL-6 with the biotinylated antibody diluent in an appropriate clean glass vial using volumes appropriate to the number of required wells. Please see example volumes below:

Number of Wells used	Biotinylated Antibody (µl)	Biotinylated Antibody Diluent (µl)
16	40	1060
24	60	1590
32	80	2120
48	120	3180
96	240	6360

8.7. Preparation of Streptavidin-HRP

It is recommended to centrifuge vial for a few seconds in a microcentrifuge to collect all the volume at the bottom.

Dilute the 5 ml vial with 0.5 ml of HRP diluent **immediately before use.** Do-not keep this diluted vial for future experiments. Further dilute the HRP solution to volumes appropriate for the number of required wells in a clean glass vial. Please see example volumes below:

Number of Wells	Streptavidin-HRP(µl)	Strep-HRP Diluent (ml)
16	30	2
24	45	3
32	60	4
48	75	5
96	150	10

10. METHOD

We strongly recommend that every vial is mixed without foaming prior to use.
Prepare all reagents as shown in section 8.
Note: final preparation of Biotinylated Secondary Antibody (section 8.6) and Streptavidin-HRP (section 8.7) should occur immediately before use.

Assay Step		**Details**
1.	**Addition**	Prepare Standard curve as shown in section 8.4 above
2.	**Addition**	Add 100 µl of each**, Sample, Standard, Control and zero (appropriate standard diluent)** in duplicate to appropriate number of wells
3.	**Addition**	Add 50 µl of diluted **biotinylated anti-IL-6** to all wells
4.	**Incubation**	Cover with a plastic plate cover and incubate at room temperature (18 to 25°C) for **3 hour(s)**
5.	**Wash**	Remove the cover and wash the plate as follows: a) Aspirate the liquid from each well b) Dispense 0.3 ml of **1x washing solution** into each well c) Aspirate the contents of each well d) Repeat step b and c another two times
6.	**Addition**	Add 100 µl of **Streptavidin-HRP** solution into all wells
7.	**Incubation**	Cover with a plastic plate cover and incubate at room temperature (18 to 25°C) for **30 min**
8.	**Wash**	Repeat wash step 5.
9.	**Addition**	Add 100 µl of ready-to-use **TMB Substrate Solution** into all wells
10.	**Incubation**	Incubate in the dark for **12-15 minutes*** at room temperature. Avoid direct exposure to light by wrapping the plate in aluminium foil.
11.	**Addition**	Add 100 µl of **H_2SO_4:Stop Reagent** into all wells
Read the absorbance value of each well (immediately after step 11.) on a spectrophotometer using 450 nm as the primary wavelength and optionally 620 nm as the reference wave length (610 nm to 650 nm is acceptable).		

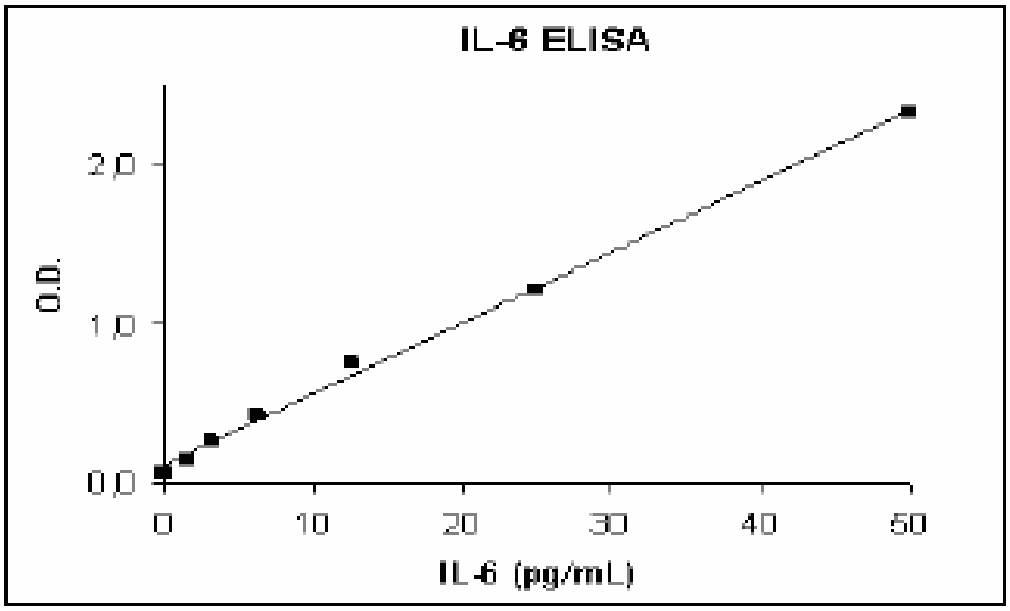
*****Incubation time of the substrate solution is usually determined by the ELISA reader performance. Many ELISA readers only record absorbance up to 2.0 O.D. Therefore the colour development within individual microwells must be observed by the analyst, and the substrate reaction stopped before positive wells are no longer within recordable range*

11. DATA ANALYSIS

Calculate the average absorbance values for each set of duplicate standards, controls and samples**.** Ideally duplicates should be within 20% of the mean.

Generate a linear standard curve by plotting the average absorbance of each standard on the vertical axis versus the corresponding IL-6 standard concentration on the horizontal axis.

The amount of IL-6 in each sample is determined by extrapolating OD values against IL-6 standard concentrations using the standard curve**.**



Example IL-6 HS Standard curve

Standard	IL-6 Conc pg/ml	OD (450 nm) mean	CV (%)
1	50	2,176	7,8
2	25	1,029	2,45
3	12,5	0,571	0,64
4	6,25	0,33	0,78
5	3,12	0,213	1,05
6	1,56	0,15	3,94
zero	0	0,069	0,02

Note: curve shown above should not be used to determine results. Every laboratory must produce a standard curve for each set of microwell strips assayed.

12. ASSAY LIMITATIONS

Do not extrapolate the standard curve beyond the maximum standard curve point. The dose-response is non-linear in this region and good accuracy is difficult to obtain. Concentrated samples above the maximum standard concentration must be diluted with Standard diluent or with your own sample buffer to produce an OD value within the range of the standard curve. Following analysis of such samples always multiply results by the appropriate dilution factor to produce actual final concentration. The influence of various drugs on end results has not been investigated. Bacterial or fungal contamination and laboratory cross-contamination may also cause irregular results. 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.
Disposable pipette tips, flasks or glassware are preferred, reusable glassware must be washed and thoroughly rinsed of all detergents before use. As with most biological assays conditions may vary from assay to assay therefore **a fresh standard curve must be prepared and run for every assay.**

13. PERFORMANCE CHARACTERISTICS

13.1 Sensitivity

The sensitivity, minimum detectable dose of IL-6 using this Diaclone IL-6 HS ELISA kit was found to be less than **0.81 pg/ml**. This was determined by adding 3 standard deviations to the mean OD obtained when the zero standard was assayed 40 times.

13.2 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-1a, IL-1b, IL-10, IL-12, IFNg, IL-4, TNFa, IL-8 and IL-13).

13.3 Precision

13.3.1 Intra-assay

Reproducibility within the assay was evaluated in three independent experiments. 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 shows 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%.**

Session	*Sample*	Assay 1 [IL-6] 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

13.3.2 Inter-assay

analysts. 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 pg/ml	Sample 2 IL-6 pg/ml	Sample 3 IL-6 pg/ml	Sample 4 IL-6 pg/ml	Sample 5 IL-6 pg/ml
A	1	24,1 23,5 23,6	10,7 10,4 10,8	5,30 5,8 5,9	44,90 36,20 39,40	27,80 30,50 31,90
	2	25,3 25,10 26,90	12,8 12,9 11,90	5,40 5,04 5,60	41,70 40,70 40,70	31,60 32,60 31,40
	3	23,13 23,72 21,97	10,50 10,42 11,09	4,99 5,22 5,59	40,06 39,67 37,93	27,01 29,99 29,95
B	1	34,46 26,70 19,69	13,60 12,55 11,69	7,47 6,97 6,04	41,98 40,48 41,97	30,94 31,85 31,68
	2	28,44 27,01 25,78	12,43 13,03 12,66	7,65 7,10 6,58	43,56 42,63 40,14	29,95 31,94 31,11
	3	25,62 24,21 24,19	11,61 10,86 11,78	6,98 6,37 6,40	44,64 38,08 37,60	31,39 29,31 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

13.4 Dilution Linearity

4 human pooled serums samples with different levels of IL-6 were analysed at different serial two fold dilutions (1:2 to 1:8) with two replicates each. Recoveries ranged from 72 to 123 % with an overall **mean recovery of 93%.**

13.5 Spiking Recovery

The spiking recovery was evaluated by spiking 3 concentrations of IL-6 into human serum in 2 separate experiments. Recoveries ranged from 107% to 123% with an overall **mean recovery of 115%.**

13.6 Stability

13.6.1 Storage Stability

Aliquots of spiked serum samples were stored at –20°C, 2-8°C, room temperature (RT) and at 37°C and the IL-6 level determined after 24h. There was no significant loss of IL-6 reactivity during storage at RT, 2-8 °C and 37 °C.

13.6.2 Freeze-thaw Stability

Aliquots of spiked serum were stored frozen at –20 °C and thawed up to 5 times and the IL-6 level was determined. There was no significant loss of IL-6 after 5 cycles of freezing and thawing.

13.7 Expected serum values

A panel of 29 human sera was tested for IL-6.
The detected IL-6 levels ranged between, below the detection level and 4.72 pg/ml with a mean level at 1.3pg/ml and a standard deviation of +/-1.4 pg/ml.

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16. ASSAY SUMMARY

Total procedure length 3 h 45 mn

Add 100 µl of sample and diluted standard/controls
and 50 µl Biotinylated anti-IL-6

⇓

Incubate 3 hours at room temperature

⇓

Wash three times

⇓

Add 100 µl of Streptavidin-HRP

⇓

Incubate 30 min at room temperature

⇓

Wash three times

⇓

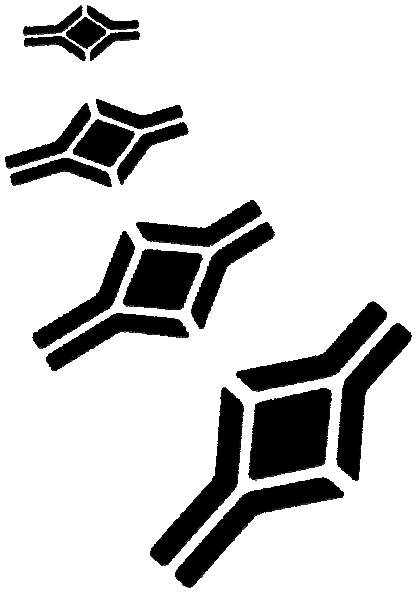
Add 100 µl of ready-to-use TMB
Protect from light. Let the color develop for 12-15 mn.

⇓

Add 100 µl H_2SO_4

⇓

Read Absorbance at 450 nm



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