Human IL-6 ELISA Kit

For the quantitative determination of human interleukin-6 (IL-6) concentrations in serum, plasma, cell culture supernatant, and other biological fluids

Catalogue Number: EL10023

96 tests

FOR LABORATORY RESEARCH USE ONLY.
NOT FOR DIAGNOSTIC USE

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S7.5 (03) IL-6

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INTENDED USE

This Human IL-6 ELISA kit is to be used for the *in vitro* quantitative determination of human interleukin-6 (IL-6) concentrations in serum, plasma, cell culture supernatant, and other biological fluids. This kit is intended FOR LABORATORY RESEARCH USE ONLY and is not for use in diagnostic or therapeutic procedures.

INTRODUCTION

Interleukin 6 (IL-6) is a multifunctional protein that plays important roles in host defense, acute phase reactions, immune responses, and hematopoiesis. [1] Synonyms for IL-6 include: B cell stimulatory factor-2 (BSF-2), [2] hybridoma/plasmacytoma growth factor, [3] hepatocyte stimulating factor, cytotoxic T cell differentiation factor and macrophage-granulocyte inducing factor 2A (MGI-2A).[1] IL-6 is expressed by a variety of normal and transformed cells including T cells, B cells, monocytes/macrophages, fibroblasts, hepatocytes, keratinocytes ,astrocytes, vascular endothelial cells, mesangial cells, osteoblasts, carcinomas, sarcomas, myelomas, glioblastomas, and melanomas. The production of IL-6 is up-regulated by numerous signals including mitogenic or antigenic stimulation, lipopolysaccharide, calcium ionophore, IL-1, IL-2, IFN, TNF, PDGF, and viruses. IL-4 and IL-13 inhibit IL-6 expression in monocytes. [4-6]

Natural human and murine IL-6 are glycoproteins containing N-and/or O-linked carbohydrates (human IL-6 contains two potential N-glycosylation sites, while mouse IL-6 has none). In comparison with mouse IL-6, human IL-6 exhibits approximately 65% sequence homology at the nucleotide level, and 42% homology at the amino acid level. Although human and mouse IL-6 are equally active on mouse cells, mouse IL-6 is not active on human cells.

IL-6 is predicted to have a four helix-bundle type tertiary structure found in a number of other cytokines including growth hormone, EPO, G-CSF, OSM, IL-11, CNTF, LIF, MGF, Prolactin, etc. [7-9] The gene structures of these cytokines also show varying degrees of relatedness. Based on these criteria, it has been suggested that these cytokines evolved from a common ancestral gene. Results of structure-function studies of IL-6 and other four α-helix bundle cytokines indicated that the c-terminal (helix D) regions of these cytokines are primarily responsible for binding to the receptors. [1. 4]

Interleukin 6 exerts multiple functions on numerous target cells. IL-6 plays an important role in immune functions. It has effects on B cell differentiation and antibody production, on cytotoxic T cell differentiation, on T cell activation, growth and differentiation, and on the induction of IL-2Rα chain expression and IL-2 production in T cells. [11-13]) In hemopoiesis, IL-6 has blast cell growth factor activity and can synergize with IL-3 to shorten the G_0 period of early hemopoietic progenitors. [14] In addition, IL-6 has been found to synergize with IL-3 in megakaryocyte development, increasing platelet numbers in vivo and the number, size and average ploidy value of megakaryocyte colonies formed from mouse or human bone marrow cells in vitro. [15-18] Similarly to IL-11 and LIF, IL-6 can induce the synthesis of hepatic acute phase proteins both in vivo and in vitro. [19] IL-6 has growth factor activities and will stimulate the growth of hybridomas, plasmacytomas, myelomas, sarcomas, [20, 21] carcinomas, [22] EBV-transformed B cells, [23] keratinocytes, and mesangial cells. In contrast to its growth stimulatory activities, IL-6 is also a growth inhibitor for a number of leukemia and carcinoma cell lines. Additional bioactivities attributed to IL-6 include: induction of terminal differentiation of M1 myeloid leukemic cells; [24] the differentiation and survival of neuronal cells; [25, 26] and the activation of osteoclast development. [10] Although IL-6 was also discovered as an antiviral factor produced by human

diploid fibroblasts, the question of whether or not IL-6 has antiviral activity is controversial. Many groups have been consistently unable to find any antiviral activity for recombinant human IL-6. [1]

The various activities of IL-6 described above suggest that this factor will have a major role in the mediation of the inflammatory and immune responses initiated by infection or injury. Although the exact functions of IL-6 in vivo are not known, elevated IL-6 levels have been reported to be associated with a variety of diseases, including auto-immune diseases, mesangial proliferative glomerulonephritis, psoriasis, and malignancies such as plasmacytoma and myeloma. A great deal of work is currently in progress in order to provide a better understanding of the role of IL-6 in the modulation of normal and pathological processes.

This IL-6 ELISA is a 3.5 hour solid phase immunoassay readily applicable to measure IL-6 levels in serum, plasma, cell culture supernatant, and other biological fluids in the range of 0 to 400 pg/mL. It showed no cross reactivity with various other cytokines superfamily proteins. This IL-6 ELISA is expected to be effectively used for further investigations into the relationship between IL-6 and various diseases.

PRINCIPLE OF THE ASSAY

This IL-6 enzyme linked immunosorbent assay (ELISA) applies a technique called a quantitative sandwich immunoassay. The microtiter plate provided in this kit has been pre-coated with a monoclonal antibody specific to IL-6. Standards or samples are then added to the appropriate microtiter plate wells with a biotin-conjugated polyclonal antibody preparation specific for IL-6 and incubated. IL-6, if present, will bind and become immobilized by the antibody pre-coated on the wells and then be “sandwiched” by biotin conjugate. The microtiter plate wells are thoroughly washed to remove unbound IL-6 and other components of the sample. In order to quantitatively determine the amount of IL-6 present in the sample, Avidin conjugated to Horseradish Peroxidase (HRP) is added to each microplate well and incubated. Avidin is a tetramer containing four identical subunits that each has a high affinity-binding site for biotin. The wells are thoroughly washed to remove all unbound HRP-conjugated Avidin and a TMB (3,3'5, 5' tetramethyl-benzidine) substrate solution is added to each well. The enzyme (HRP) and substrate are allowed to react over a short incubation period. Only those wells that contain IL-6, biotin-conjugated antibody and enzyme-conjugated Avidin will exhibit a change in colour. The enzyme-substrate reaction is terminated by the addition of a sulphuric acid solution and the colour change is measured spectrophotometrically at a wavelength of 450nm ± 2 nm.

In order to measure the concentration of IL-6 in the samples this kit includes two calibration diluents (Calibrator Diluent I for serum/plasma testing and Calibrator Diluent II for cell culture supernatant testing.) According to the testing system, the provided standard is diluted (2-fold) with the appropriate Calibrator Diluent and assayed at the same time as the samples. This allows the operator to produce a standard curve of Optical Density (O.D) versus IL-6 concentration (pg/mL). The concentration of IL-6 in the samples is then determined by comparing the O.D. of the samples to the standard curve.

LIMITATIONS OF THE PROCEDURE

- FOR LABORATORY RESEARCH USE ONLY, NOT FOR USE IN DIAGNOSTIC PROCEDURES.

- As manufacturers we take great care to ensure that our products are suitable for use with all validated sample types, as designated in the product insert. However, it is possible that in some cases, high levels of interfering factors may cause unusual results.
- The kit should not be used beyond the expiration date on the kit label.
- It is important that the Calibrator Diluent selected for the standard curve be consistent with the samples being assayed.
- If samples generate values higher than the highest standard, dilute the samples with the appropriate Calibrator Diluent and repeat the assay.
- Any variation in standard diluent, operator, pipetting technique, washing technique, incubation time or temperature, and kit age can cause variation in binding.
- Soluble receptors or other binding proteins present in biological samples do not necessarily interfere with the measurement of ligands in samples. However, until the factors have been tested, the possibility of interference cannot be excluded.

REAGENTS PROVIDED

All reagents provided are stored at 2-8°C. Refer to the expiration date on the label.

	96 tests

1. **IL-6 MICROTITER PLATE** (Part EL23-1) ***96 wells***
   Pre-coated with anti-human IL-6 monoclonal antibody.

2. **BIOTIN CONJUGATE** (Part EL23-2) ***6 mL***
   Anti-human IL-6 monoclonal antibody conjugated to Biotin.

3. **AVIDIN CONJUGATE** (Part EL23-3) ***12 mL***
   Avidin conjugated to horseradish peroxidase.

4. **IL-6 STANDARD** (Part EL23-4) ***2 vials***
   Recombinant human IL-6 (800 pg/vial) in a buffered protein base with preservative, lyophilized.

5. **CALIBRATOR DILUENT I** (Part EL23-5) ***25 mL***
   Animal serum with buffer and preservative. *For serum/plasma testing.*

6. **CALIBRATOR DILUENT II (5X)** (Part EL23-6) ***25 mL***
   Cell culture medium with calf serum and preservative. *For cell culture supernatant testing.*

7. **WASH BUFFER (20X)** (Part 30005) ***60 mL***
   20-fold concentrated solution of buffered surfactant.

8. **SUBSTRATE A** (Part EL23-7) ***10 mL***
   Buffered solution with H_2O_2

9. **SUBSTRATE B** (Part 30007) ***10 mL***
   Buffered solution with TMB.

10. **STOP SOLUTION** (Part 30008) ***14 mL***
    2N Sulphuric Acid (H_2SO_4). Caution: Caustic Material!

MATERIALS REQUIRED BUT NOT SUPPLIED

1. Single or multi-channel precision pipettes with disposable tips: 10-100μL and 50-200μL for running the assay.
2. Pipettes: 1 mL, 5 mL 10 mL, and 25 mL for reagent preparation.
3. Multi-channel pipette reservoir or equivalent reagent container.
4. Test tubes and racks.
5. Polypropylene tubes or containers (25 mL).
6. Erlenmeyer flasks: 100 mL, 400 mL, 1 L and 2 L.
7. Microtiter plate reader (450 nm ± 2nm)
8. Automatic microtiter plate washer or squirt bottle.
9. Sodium hypochlorite solution, 5.25% (household liquid bleach).
10. Deionized or distilled water.
11. Plastic plate cover.
12. Disposable gloves.
13. Absorbent paper.

PRECAUTIONS

1. Do not substitute reagents from one kit lot to another. Standard, conjugate and microtiter plates are matched for optimal performance. Use only the reagents supplied by manufacturer.
2. Allow kit reagents and materials to reach room temperature (20-25°C) before use. Do not use water baths to thaw samples or reagents.
3. Do not use kit components beyond their expiration date.
4. Use only deionized or distilled water to dilute reagents.
5. Do not remove microtiter plate from the storage bag until needed. Unused strips should be stored at 2-8°C in their pouch with the desiccant provided.
6. Use fresh disposable pipette tips for each transfer to avoid contamination.
7. Do not mix acid and sodium hypochlorite solutions.
8. Human serum and plasma should be handled as potentially hazardous and capable of transmitting disease. Disposable gloves must be worn during the assay procedure, since no known test method can offer complete assurance that products derived from human blood will not transmit infectious agents. Therefore, all blood derivatives should be considered potentially infectious and good laboratory practices should be followed.
9. All samples should be disposed of in a manner that will inactivate human viruses.
   Solid Wastes: Autoclave 60 min. at 121°C.
   Liquid Wastes: Add sodium hypochlorite to a final concentration of 1.0%. The waste should be allowed to stand for a minimum of 30 minutes to inactivate the virus before disposal.
10. Substrate Solution is easily contaminated. If bluish prior to use, *do not use*.
11. Substrate B contains 20% acetone, keep this reagent away from sources of heat or flame.
12. If Wash Buffer (20X) is stored at a lower temperature (2-5°C), crystals may form, which must be dissolved by warming to 37°C prior to use.

SAMPLE PREPARATION

1. COLLECTION, HANDLING, AND STORAGE

a) **Cell Culture Supernatant:** Centrifuge to remove any visible particulate material.
b) **Serum**: Blood should be drawn using standard venipuncture techniques and serum separated from the blood cells as soon as possible. Samples should be allowed to clot for one hour at room temperature, centrifuged for 10 minutes (4°C), and serum extracted.
c) **Plasma:** Blood should be drawn using standard venipuncture techniques and plasma collected using sodium citrate, EDTA, or heparin as an anticoagulant. To ensure optimal recovery and minimal platelet contamination, after collection there must be quick separation of plasma with less than 30 minutes on ice. Centrifuge for 10 minutes (4°C) to remove any particulates.

- Avoid grossly hemolytic, lipidic or turbid samples.
- Serum, plasma, cell culture supernatant, and urine samples to be used within 24-48 hours may be stored at 2-8°C, otherwise samples must stored at -20°C to avoid loss of bioactivity and contamination. Avoid freeze-thaw cycles.
- When performing the assay slowly bring samples to room temperature.
- It is recommended that all samples be assayed in duplicate.
- DO NOT USE HEAT-TREATED SPECIMENS.

PREPARATION OF REAGENTS

Remove all kit reagents from refrigerator and allow them to reach room temperature (20-25°C). Prepare the following reagents as indicated below. Mix thoroughly by gently swirling before pipetting. Avoid foaming.

1. **Calibrator Diluent II (1X):** Mix well before diluent. Add 1 volume of Calibrator Diluent II (5x) to 4 volumes of distilled or deionized water. Mix well before use.

2. **Wash Buffer (1X):** Add 60 mL of Wash Buffer (20X) and dilute to a final volume of 1200 mL with distilled or deionized water. Mix thoroughly. If a smaller volume of Wash Buffer (1X) is desired, add 1 volume of Wash Buffer (20X) to 19 volumes of distilled or deionized water. Wash Buffer (1X) is stable for 1 month at 2-8°C. Mix well before use.

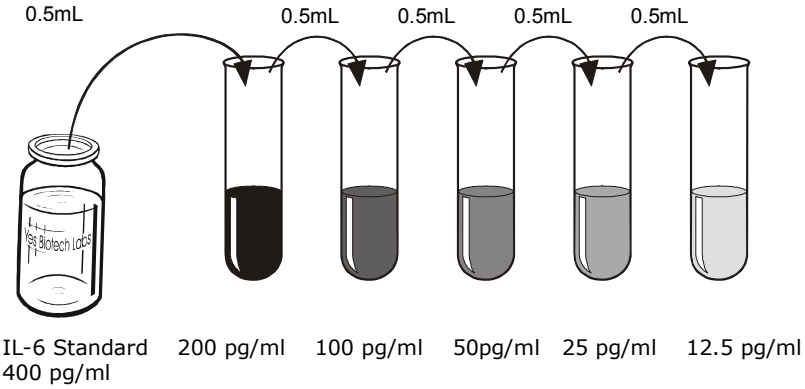
3. **Substrate Solution:** Substrate A and Substrate B should be mixed together in equal volumes up to 15 minutes before use. Refer to the table below for correct amounts of Substrate Solution to prepare.

Strips Used	Substrate A (mL)	Substrate B (mL)	Substrate Solution (mL)
2 strips (16 wells)	1.5	1.5	3.0
4 strips (32 wells)	3.0	3.0	6.0
6 strips (48 wells)	4.0	4.0	8.0
8 strips (64 wells)	5.0	5.0	10.0
10 strips (80 wells)	6.0	6.0	12.0
12 strips (96 wells)	7.0	7.0	14.0

4. **<u>IL-6 Standard</u>:**

a) Two vials of Standards are provided in this kit to allow both serum/plasma and cell culture supernatent testing. Reconstitute the IL-6 Standard with either 2.0 mL of Calibrator Diluent I (for serum/plasma testing) or Calibrator Diluent II (for cell culture supernatant testing). This reconstitution produces a stock solution of 400 pg/mL. Allow solution to sit for at least 15 minutes with gentle agitation prior to making dilutions. Use within one hour of reconstituting. The IL-6 standard stock solution can be stored frozen (-20°C) for up to 30 days. Avoid freeze-thaw cycles; aliquot if repeated use is expected.

b) Use the above stock solution to produce a serial 2-fold dilution series within the range of this assay (12.5 pg/mL to 400 pg/mL) as illustrated. Add 0.5 mL of the <u>appropriate Calibrator Diluent</u> to each test tube. Between each test tube transfer be sure to mix contents thoroughly. The undiluted IL-6 Standard will serve as the **high standard (400 pg/mL**) and the Calibrator Diluent will serve as the zero standard (0 pg/mL).



ASSAY PROCEDURE

1. Prepare Wash Buffer (1X) and IL-6 Standards before starting assay procedure (see Preparation of Reagents). *It is recommended that the table and diagram provided be used as a reference for adding Standards or Samples to the Microtiter Plate.*

Wells	Contents			**Wells**	Contents		
1A, 1B	Standard 1	0 **pg/mL**	(S1)	**2A, 2B**	Standard 5	100 **pg/mL**	(S5)
1C, 1D	Standard 2	12.5**pg/mL**	(S2)	**2C, 2D**	Standard 6	200 **pg/mL**	(S6)
1E, 1F	Standard 3	25 **pg/mL**	(S3)	**2E, 2F**	Standard 7	400 **pg/mL**	(S7)
1G, 1H	Standard 4	50 **pg/mL**	(S4)	**2G, 12H**	IL-6 samples		

	1	2	3	4	5	6	7	8	9	10	11	12
A	S1	S5	2	6	10	14	18	22	26	30	34	38
B	S1	S5	2	6	10	14	18	22	26	30	34	38
C	S2	S6	3	7	11	15	19	23	27	31	35	39
D	S2	S6	3	7	11	15	19	23	27	31	35	39
E	S3	S7	4	8	12	16	20	24	28	32	36	40
F	S3	S7	4	8	12	16	20	24	28	32	36	40
G	S4	1	5	9	13	17	21	25	29	33	37	41
H	S4	1	5	9	13	17	21	25	29	33	37	41

2. Add 100μL of Standard or Sample to the appropriate well of the antibody pre-coated Microtiter Plate and incubate 1 hour at room temperature.

3. Without discarding the standards and samples, add 50μL Anti-IL6 Biotin conjugate to each wells. Mix well. Cover and incubate for 1 hour at room temperature.

4. Wash the Microtiter Plate using one of the specified methods indicated below:

   Manual Washing: Remove incubation mixture by aspirating contents of the plate into a sink or proper waste container. Using a squirt bottle, fill each well completely with Wash Buffer (1X) then aspirate contents of the plate into a sink or proper waste container. Repeat this procedure four more times for a **total of FIVE washes**. After final wash, invert plate, and blot dry by hitting plate onto absorbent paper or paper towels until no moisture appears. *Note*: Hold the sides of the plate frame firmly when washing the plate to assure that all strips remain securely in frame.

   Automated Washing: Aspirate all wells, then wash plates **FIVE times** using Wash Buffer (1X). Always adjust your washer to aspirate as much liquid as possible and set fill volume at 350 μL/well/wash (range: 350-400 μL). After final wash, invert plate, and blot dry by hitting plate onto absorbent paper or paper towels until no moisture appears. *It is recommended that the washer be set for a soaking time of 10 seconds or shaking time of 5 seconds between washes.*

5. Dispense 100μl of Avidin Conjugate to each well Mix well. Cover and incubate for 1 hour at room temperature.

6. Prepare Substrate Solution no more than 15 minutes before end of second incubation (see Preparation of Reagents).

7. Repeat wash procedure as described in Step 4.

8. Add 100μL Substrate Solution to each well. Cover and incubate for 15 minutes at room temperature.

9. Add 100μL Stop Solution to each well. Mix well.

10. Read the Optical Density (O.D.) at 450 nm using a microtiter plate reader set within 30 minutes.

CALCULATION OF RESULTS

The standard curve is used to determine the amount of IL-6 in an unknown sample. The standard curve is generated by plotting the average O.D. (450 nm) obtained for each of the standard concentrations on the vertical (Y) axis versus the corresponding IL-6 concentration (pg/mL) on the horizontal (X) axis.

1. First, calculate the mean O.D value for each standard and sample. All O.D. values are subtracted by the value of the zero-standard (0 pg/mL) before result interpretation. Construct the standard curve using graph paper or statistical software.

2. To determine the amount of IL-6 in each sample, first locate the O.D. value on the Y-axis and extend a horizontal line to the standard curve. At the point of intersection, draw a vertical line to the X-axis and read the corresponding IL-6 concentration. If samples generate values higher than the highest standard, dilute the samples with the appropriate Calibrator Diluent and repeat the assay, the concentration read from the standard curve must be multiplied by the dilution factor.

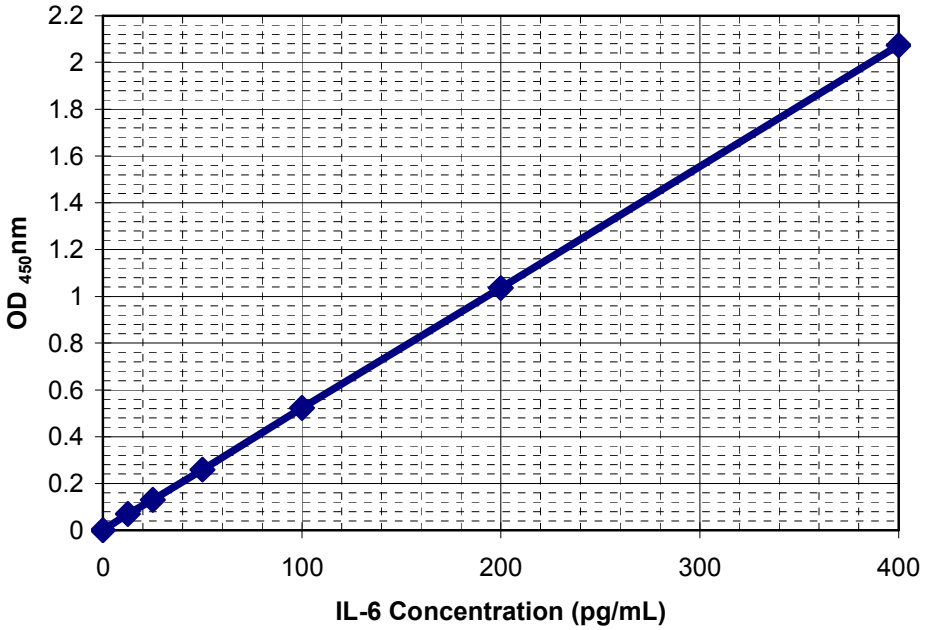
TYPICAL DATA

Results of a typical standard run of a IL-6 ELISA are shown below. Any variation in standard diluent, operator, pipetting and washing technique, incubation time or temperature, and kit age can cause variation in result. The following examples are for the purpose of illustration only, and should not be used to calculate unknowns. Each user should obtain his or her own standard curve

EXAMPLE ONE

The following data was obtained for a standard curve using Calibrator Diluent I.

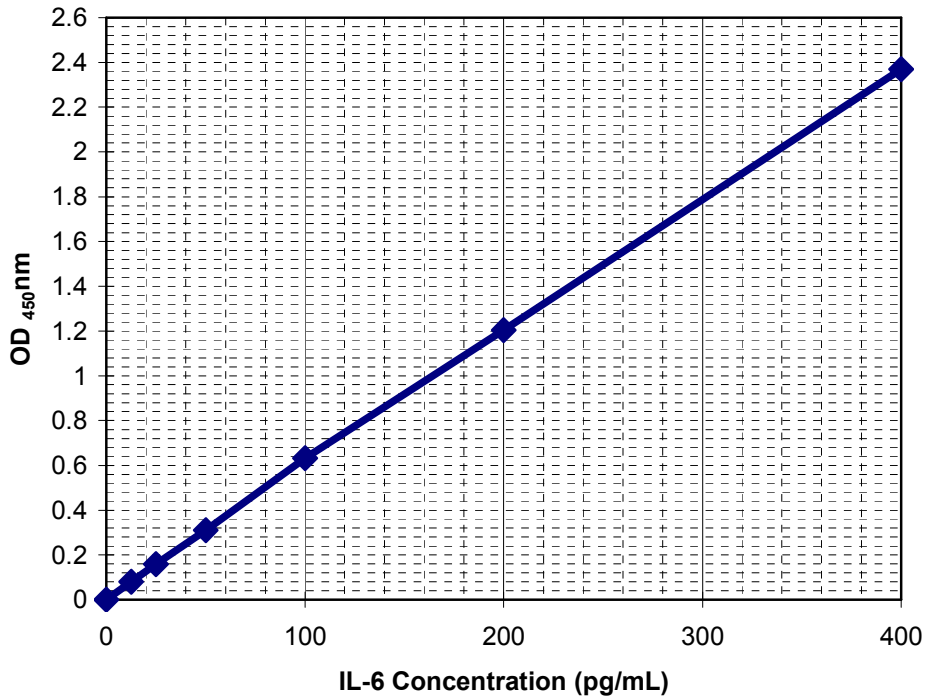
Standard (pg/mL)	O.D. (450 nm)	Mean	Zero Standard Subtracted (Std.)-(S1)
0	0.050, 0.048	0.049	0
12.5	0.115, 0.120	0.118	0.069
25	0.178, 0.180	0.179	0.130
50	0.310, 0.305	0.308	0.259
100	0.570, 0.574	0.572	0.523
200	1.080, 1.088	1.084	1.035
400	2.120, 2.126	2.123	2.074



EXAMPLE TWO

The following data was obtained for a standard curve using Calibrator Diluent II.

Standard (pg/mL)	O.D. (450 nm)	Mean	Zero Standard Subtracted (Std.) -(S1)
0	0.050, 0.052	0.051	0
12.5	0.130, 0.134	0.132	0.081
25	0.210, 0.208	0.209	0.158
50	0.360, 0.364	0.362	0.311
100	0.680, 0.685	0.683	0.632
200	1.250, 1.260	1.255	1.204
400	2.420, 2430	2.425	2.374



PERFORMANCE CHARACTERISTICS

1. INTRA-ASSAY PRECISION

To determine within-run precision, three different samples of known concentration were assayed by replicates of 20 in 1 assay.

	Calibrator Diluent I assay			*Calibrator Diluent II assay*		
Sample	**1**	**2**	***3***	**1**	**2**	**3**
n	20	20	20	20	20	20
Mean (pg/mL)	30.9	121.3	353.8	24.1	100.3	307.9
Standard Deviation (pg/mL)	2.7	6.3	15.6	1.47	5.08	16.2
Coefficient of Variation (%)	*8.7*	*5.2*	*4.4*	*6.1*	*5.1*	*5.2*

2. INTER-ASSAY PRECISION

To determine between-run precision, three different samples of known concentration were assayed by replicates on 20 different assays.

	Calibrator Diluent I assay			*Calibrator Diluent II assay*		
Sample	**1**	**2**	**3**	**1**	**2**	**3**
n	20	20	20	20	20	20
Mean (pg/mL)	30.5	132.7	461.1	23.9	97.5	270.0
Standard Deviation (pg/mL)	2.26	7.62	20.6	1.98	4.16	12.9
Coefficient of Variation (%)	*7.4*	*5.7*	*4.5*	*8.3*	*4.3*	*4.8*

3. **RECOVERY**
The recovery of IL-6 spiked to 3 different levels throughout the range of the assay in various matrices was evaluated.

Sample Type	Average Recovery %	Range %
Cell culture media	97	89 - 101
Serum	95	85 - 108
Plasma	90	80 - 95

4. **SENSITIVITY**
The minimum detectable dose of IL-6 was determined by adding two standard deviations to the mean optical density value of the zero standard replicates and calculating the corresponding concentration from the standard curve. The minimum detectable dose using a standard curve generated with Calibrator Diluent I is 2.0 *pg/mL* and using Calibrator Diluent II is 1.75 *pg/mL*.

5. **SPECIFICITY**
This sandwich ELISA recognizes both natural and recombinant human IL-6. This kit exhibits no detectable cross-reactivity with human; SAA, EGF, IL-8, IL-16, MCAF, MCP-3, TNF-γ, M-CSF, GM-CSF, TGF-ß1, RANTES, FGF, MIP-1α, CRP, EPO, IFN-γ.

6. **CALIBRATION**
This immunoassay is calibrated against WHO Standard (First International Standard, 1992. Code No.: 89/548.

7. **EXPECTED NORMAL VALUES**
Biological samples from apparently healthy, normal individuals were collected and the average IL-6 concentration measured. Serum/plasma samples (n=17) averaged less than 12.5 pg/mL, so did the urine samples.

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