Product Manual

IL-6 (human), high sensitivity ELISA Kit

Catalog #: ENZ-KIT178-0001

96-Well Kit

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Product Manual

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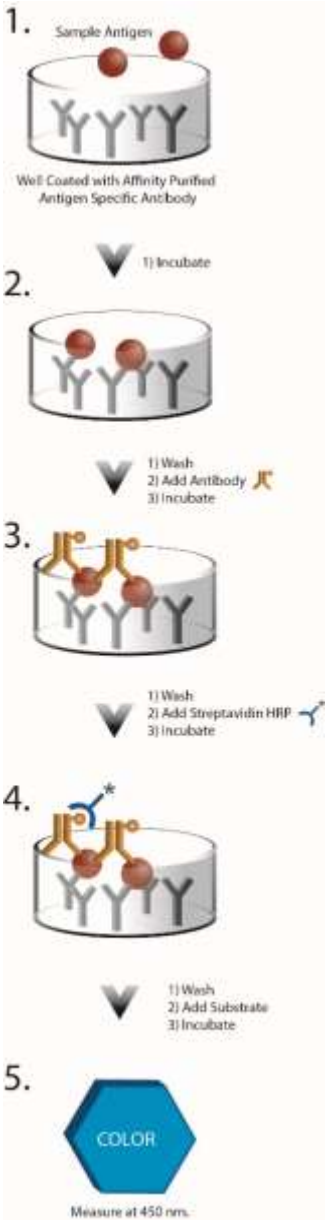
Please read entire booklet before proceeding with the assay.

INTRODUCTION

The IL-6 (human) high sensitivity ELISA kit is a complete immunometric RUO (research use only) kit for the quantitative determination of IL-6 in human serum, plasma, urine, and tissue culture media. Please read the complete kit insert before performing this assay.

Interleukin-6 (IL-6) is a pleiotropic pro-inflammatory cytokine, involved in the induction, growth, and differentiation of cells in the immune and hematopoietic systems, and initiates and coordinates inflammatory reaction[1]. Formerly known as B-cell differentiation factor[2], IL-6 is a 24 kDa protein that is 212 amino acids in length. It is produced by various types of cells, such as macrophages, T-cells, B-cells and fibroblasts[3]. IL-6 is one of the essential factors for antibody production in B cells, a potent growth factor for myeloma and plasmocytoma cells, and induces acute phase proteins in hepatocytes and promotes expansion of activated T-cells[4]. Levels of IL-6 have been shown to increase in serum as a result of burn trauma and sepsis and also in cerebrospinal fluid with acute viral or bacterial infections of the central nervous system (CNS)[5]. Increased levels of IL-6 are observed in several inflammatory diseases, such as rheumatoid arthritis, Castleman's disease and systemic juvenile idiopathic arthritis as well as playing a critical role in the pathogenesis of autoimmune disease[6]. In addition to all of the functions described above, IL-6 also acts as a myokine, which is a cytokine produced by working muscle during exercise[7].



PRINCIPLE

1. The kit uses a monoclonal antibody specific to IL-6 immobilized on a microtiter plate. Standards or samples containing IL-6 are added to the plate.
2. After a short incubation the excess sample or standard is washed out and a biotinylated monoclonal antibody to IL-6 is added. This antibody binds to the IL-6 captured on the plate.
3. After a short incubation the excess antibody is washed out and Streptavidin conjugated to Horseradish peroxidase (SA-HRP) is added, which binds to the biotin on the bound monoclonal antibody. The plate is then incubated.
4. Once the incubation is complete, excess conjugate is washed out and TMB substrate solution is added and incubated. An HRP-catalyzed reaction generates a blue color in the solution.
5. Stop solution is added to stop the substrate reaction. The resulting yellow color is read at 450 nm. The amount of signal is directly proportional to the level of IL-6 in the sample.

MATERIALS SUPPLIED

1. **IL-6 Microtiter Plate, One Plate of 96 Wells**
   Catalog No. 80-2772

   A plate using break-apart strips coated with a monoclonal antibody to IL-6.

2. **Assay Buffer, 27 mL**
   Catalog No. 80-0010

   Tris buffered saline containing proteins and detergents.

Do not mix components from different kit lots or use reagents beyond the expiration date of the kit.

3. **IL-6 Antibody, 10x Concentrate**
   Catalog No. 80-2773-0001

   Monoclonal antibody specific to IL-6.

4. **Antibody Diluent, 14 mL**
   Catalog No. 80-2722

   Buffer used to dilute the IL-6 antibody concentrate.

5. **IL-6 Standard, 10x Concentrate**
   Catalog No. 80-2771

   Two vials containing 500 pg/mL IL-6 each.

6. **Wash Buffer Concentrate, 100 mL**
   Catalog No. 80-1287

   One bottle containing 20x Tris buffered saline with detergent.

7. **IL-6 Conjugate, 10 mL**
   Catalog No. 80-2774-0010

   A solution of Streptavidin-conjugated Horseradish Peroxidase.

Protect substrate from prolonged exposure to light.

8. **TMB Substrate, 25 mL**
   Catalog No. 80-2101

   A solution of 3,3'5,5' tetramethylbenzidine (TMB) and hydrogen peroxide.

Stop solution is caustic. Keep tightly capped.

9. **Stop Solution 2, 10 mL**
   Catalog No. 80-0377

   A 1N solution of hydrochloric acid in water.

10. **IL-6 Assay Layout Sheet, 1 each**
    Catalog No. 30-0349

11. **Plate Sealer, 3 each**
    Catalog No. 30-0012

This kit uses break-apart microtiter strips, which allow the user to measure as many samples as desired. Unused wells must be kept desiccated at 4°C in the sealed bag provided. The wells should be used in the frame provided.

STORAGE

All kit components, except for the standard, are stable at 4°C until the kit's expiration date. Both vials of the 10x IL-6 standard should be stored at -20°C upon receipt. Shipping conditions may not reflect storage conditions.

OTHER MATERIALS NEEDED

1. Deionized or distilled water
2. Precision pipets for volumes between 5 µL and 1,000 µL
3. Repeater pipet for dispensing 100 µL
4. Disposable beakers for diluting buffer concentrates
5. Benchtop centrifuge
6. Graduated cylinders
7. A microplate shaker
8. Adsorbent paper for blotting
9. Microplate reader capable of reading a 450 nm
10. Software for extrapolating sample values from optical density readings utilizing a four parameter logistic curve fit.

SAMPLE HANDLING

Samples must be stored at or below -20°C to avoid loss of bioactive analyte. Avoid repeated freeze/ thaw cycles.

If buffers other than those provided are used in the assay, the end-user must determine the appropriate dilution and assay validation.

The IL-6 high sensitivity ELISA is suitable for measuring IL-6 in human serum, EDTA plasma and urine as well as tissue culture media. Prior to sample analysis in the assay, frozen samples should be slowly thawed at room temperature and vortexed to mix. Samples diluted sufficiently into assay buffer can be read directly from a standard curve. **Both serum and plasma must be centrifuged at 10,000 rpm for 1 minute prior to diluting to at least 1:8 with assay buffer.** Both of these actions will remove matrix interference effects (please refer to the Spike and Recovery section on page 6 for detailed data). Neat urine and tissue culture media have also been validated for use in this assay. The minimum recommended dilution may not be optimal for all samples for the levels of endogenous IL-6 could vary between sample groups. Therefore, it is up to each end user to optimize the dilution for their unique set of samples.

SAMPLE MATRIX PROPERTIES

Linearity

Three pools of human serum with biologically relevant levels of IL-6 were serially diluted in assay buffer and run in the assay. Additionally, three pools of human EDTA plasma were diluted to the MRD (1:8), spiked with human IL-6 and diluted in assay buffer. The data was compared to the standard curve. The results are shown in the table below.

Dilutional Linearity, %			
Dilution	**EDTA Plasma pool 1**	**EDTA Plasma pool 2**	**EDTA Plasma pool 3**
1:8	100	100	100
1:16	83	83	84
1:32	72	71	58
1:64	68	79	90

Dilutional Linearity, %			
Dilution	**Serum pool 1**	**Serum pool 2**	**Serum pool 3**
1:8	100	100	100
1:16	88.6	68.4	75
1:32	97.4	98.6	98.6
1:64	97.4	120.7	118.3
1:128	105.6	125.5	125.9
1:256	--	69.2	81.2

Spike and Recovery

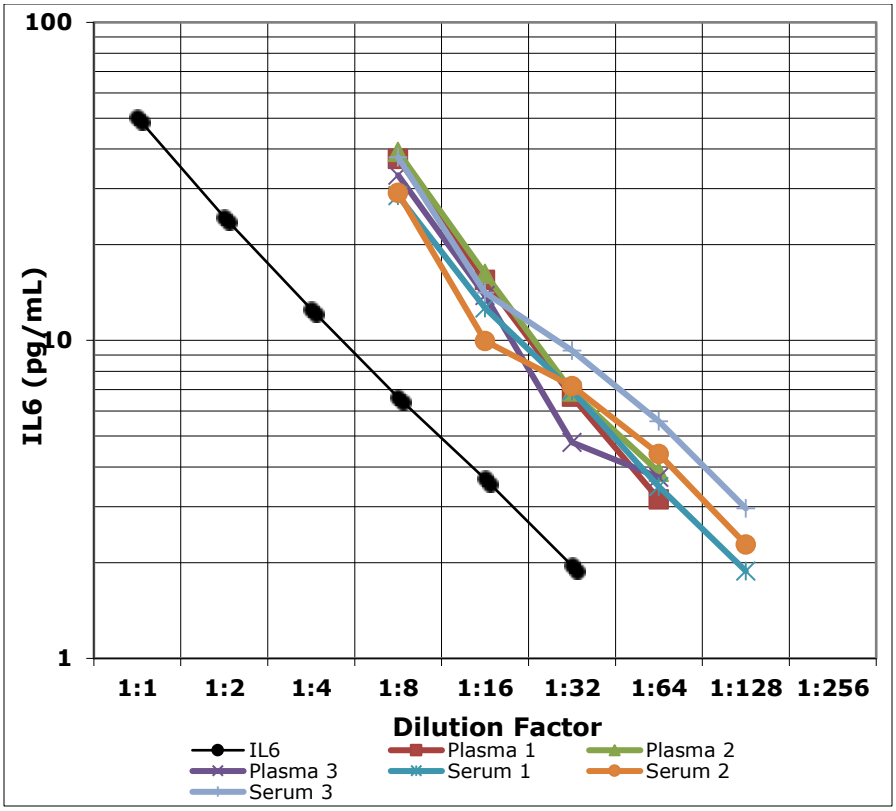
Three pools of human serum and an additional three pools of EDTA plasma were diluted 1:8 in assay buffer and then spiked with human IL-6 at a high, medium and low concentration. Neat human urine and tissue culture media were also spiked with the same levels of IL-6. All samples were run in the assay. Matrix background was subtracted and the percent recovery of the spiked IL-6 was determined.

Sample	Spike Concentration, pg/mL	% Recovery	Minimum Recommended Dilution
EDTA Plasma pool 1	35	82.9	1:8
	15	97.6	
	6	135.2	
EDTA Plasma pool 2	35	84.9	1:8
	15	92.1	
	6	135.4	
EDTA Plasma pool 3	35	107.5	1:8
	15	101.3	
	6	147.3	
Serum pool 1	35	98.1	1:8
	15	87.4	
	6	118.4	
Serum pool 2	35	94.3	1:8
	15	76.4	
	6	110	
Serum pool 3	35	70.3	1:8
	15	68	
	6	79.1	

Sample	Spike Concentration, pg/mL	% Recovery	Minimum Recommended Dilution
Urine	35	99.5	Neat
	15	103	
	6	112	
Tissue Culture Media	35	107.1	Neat
	15	119	
	6	109.7	

Parallelism

To assess parallelism, three lots of human serum with biologically relevant levels of IL-6 were diluted to the MRD of 1:8, serially diluted in assay buffer and then run in the assay. Additionally, three lots of human EDTA plasma were diluted to the MRD (1:8), spiked with human IL-6 and serially diluted in assay buffer. The IL-6 concentration in each sample was assigned using the standard curve. Assigned concentrations were plotted as a function of sample dilution. Parallelism of the curves demonstrates that the antigen binding characteristics are similar enough to allow the accurate determination of native analyte levels in diluted samples of human origin.



SAMPLE TESTING

Seventeen human serum samples were first centrifuged for one minute at 10,000 rpm, diluted 1:8 in assay buffer and run in the assay. IL-6 was detected in all samples tested and endogenous levels ranged from 3.3-562.4 pg/mL IL-6.

# of Samples tested	Type of Sample	IL-6 range, pg/mL
17	Human serum	3.3-562.4

Sample handling procedures should be completed prior to reagent preparation.

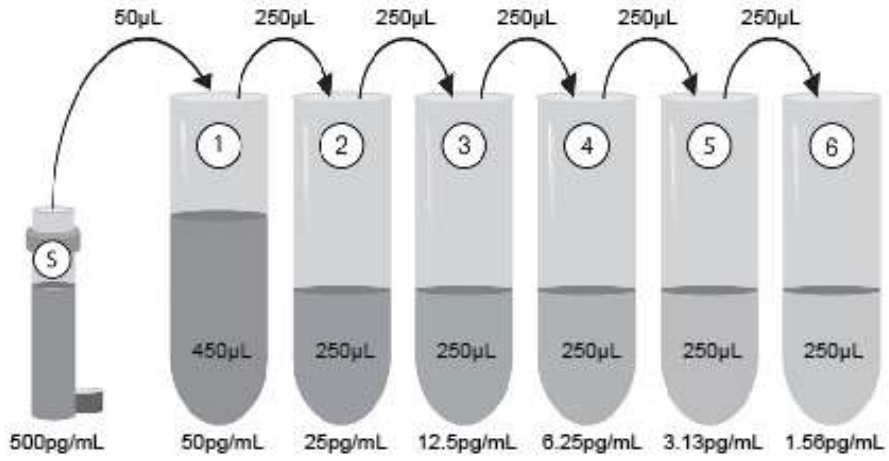
REAGENT PREPARATION

1. **Wash Buffer**

   Prepare Wash buffer by diluting 50 mL of the supplied Wash Buffer concentrate with 950 mL of deionized water. The diluted wash buffer can be stored at room temperature for up to 3 months.

2. **IL-6 Standard**

   Remove IL-6 standard from -20°C and allow to thaw at room temperature. Label six 12x75 mm polypropylene tubes #1 through #6. Add 450 µL of Assay Buffer into tube #1. Add 250 µL of Assay Buffer into tube #2 through tube #6. Add 50 µL of 10x IL-6 standard stock to tube #1 and vortex gently. Add 250 µL of tube #1 into tube #2 and vortex. Add 250 µL of tube #2 to tube #3 and vortex thoroughly. Continue this for tubes #4 through #6.



Diluted standards should be used within 60 minutes of preparation. Please note that standards provided are one-time use aliquots. Discard any unused standard dilutions and 10x standard concentrate.

3. **IL-6 Antibody**

   The IL-6 antibody is supplied as a 10x concentrate. Dilute appropriate volume to 1x (i.e. 0.5 mL 10x stock into 4.5 mL Antibody Diluent) and store the unused 10x concentrate at 4ºC.

ASSAY PROCEDURE

Refer to the Assay Layout Sheet to determine the number of wells to be used. Remove unneeded wells and return them, with the desiccant, to the plate bag and seal. Store the unused wells at 4°C.

Bring all reagents to room temperature for at least 30 minutes prior to opening.

1. Add 100 µL of Assay Buffer into the S0 (0 pg/mL standard) and NSB (non-specific binding) wells. Leave the Blank wells empty.

All standards and samples should be run in duplicate.

2. Add 100 µL of standards #1 through #6 into the appropriate wells.
3. Add 100 µL of the samples into the appropriate wells.
4. Seal the plate. Incubate at room temperature (RT) on a plate shaker for 1 hour at ~500 rpm*****. **See note.**

Pipet the reagent to the side of the wells to avoid possible contamination.

5. Empty the contents of the wells and wash by adding a full well volume (~400 µL) of 1X Wash Buffer to each well. Empty or aspirate the wells and repeat the wash 2 more times for a total of 3 washes. After the final wash, empty or aspirate the wells and firmly tap the plate on a lint free paper towel to remove any remaining wash buffer.
6. Add 100 µL of 1X IL-6 Antibody into each well, except the NSB and Blank. Add 100 µL Antibody Diluent into NSB wells and leave the Blank wells empty.
7. Seal the plate. Incubate at RT on a plate shaker for 1 hour at ~500 rpm.
8. Wash as above (Step 5).

9. Add 100 µL of IL-6 Conjugate to each well, except the Blank wells.
10. Seal the plate and incubate at RT on a plate shaker for 30 minutes at ~500 rpm.
11. Wash as above (Step 5).
12. Add 100 µL of TMB substrate into all wells.
13. Seal the plate and incubate at RT on a plate shaker for 30 minutes at ~500 rpm.
14. Add 100 µL of Stop Solution into each well.
15. After zeroing the plate reader against the Blank, read optical density at 450 nm. If the plate reader is not capable of adjusting for the blank, manually subtract the mean OD of the substrate blank from all readings.

*** Note: The plate shaker speed was based on a BellCo Mini Orbital Shaker (mod no. 7744-08096). The actual speed of the plate shaker should be such that the liquid in the plate wells mixes thoroughly, but does not splash out of the well.**

CALCULATION OF RESULTS

Make sure to multiply sample concentrations by the dilution factor used during sample preparation.

Several options are available for the calculation of the concentration of IL-6 in the samples. We recommend that the data be handled by an immunoassay software package utilizing a 4 parameter logistic (4PL) curve fitting program. The concentration of IL-6 can be calculated as follows:

1. Calculate the average net OD for each standard and sample by subtracting the average NSB OD from the average OD for each standard and sample.

   Average Net OD = Average OD - Average NSB OD

2. Using data analysis software, plot the Average Net OD for each standard versus IL-6 concentration in each standard. Samples with concentrations outside of the standard curve range will need to be reanalyzed using alternative dilution(s).

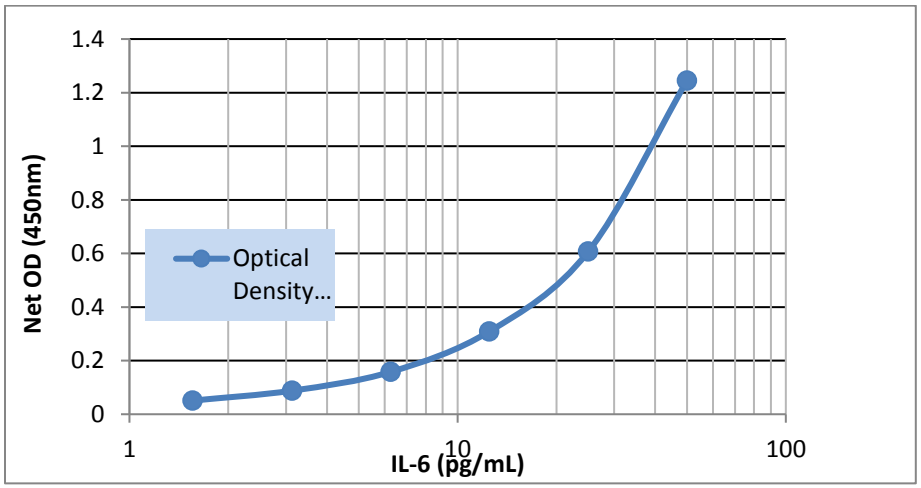
TYPICAL RESULTS

The results shown below are for illustration only and should not be used to calculate results.

Sample	Mean OD	Net OD	IL-6 (pg/mL)
NSB	0.045	--	--
S0	0.060	0.015	0
S1	1.290	1.245	50
S2	0.652	0.607	25
S3	0.354	0.309	12.5
S4	0.203	0.158	6.25
S5	0.133	0.088	3.13
S6	0.096	0.051	1.56

Typical Standard Curve

A typical standard curve is shown below. This curve must not be used to calculate IL-6 concentrations; each user must run a standard curve for each assay.



PERFORMANCE CHARACTERISTICS

Specificity

The specificity of the assay was determined by running serial dilutions of the analytes, including the cross-reactant, in the assay, fitting the resulting dose response curve(s) to a 4PL curve-fit and determining the ED_{50}. The ED_{50} of the cross-reactant was then divided by the determined ED_{50} of the IL-6 standard curve and multiplied by 100.

Analyte	Cross Reactivity
IL-6	100%
IL-1α	0.068%
IL-1β	0.00%
IL-2	0.00%
IL-3	0.286%
IL-4	0.014%
IL-7	2.36%
IL-8	0.338%
TNFα	0.126%
TNFβ	0.091%

Sensitivity

The sensitivity or limit of detection of the assay is 0.057 pg/mL and was determined by interpolation at 2 standard deviations above the background (0 pg/mL) of 16 zero standard replicates. Data was used from 19 standard curves.

Intra-assay precision was determined by assaying 20 replicates of three controls containing IL-6 in a single assay.

Intra-assay precision	
pg/mL	**%CV**
20.88	4.38
9.94	4.08
4.38	4.06

Inter-assay precision was determined by measuring controls of varying IL-6 concentrations in multiple assays over several days.

Inter-assay precision	
pg/mL	**%CV**
21.9	9.6
10.2	9.3
4.8	9.4

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