



## **TiterZyme<sup>®</sup> EIA**

### **human IL-6**

# **Enzyme Immunometric Assay Kit**

**Catalog No. 900-033**

### **96 Well Kit**

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**FOR RESEARCH USE ONLY. NOT FOR USE IN DIAGNOSTIC PROCEDURES.**

## **Description**

Assay Designs' human IL-6 TiterZyme® Enzyme Immunometric Assay (EIA) kit is a complete kit for the quantitative determination of human IL-6 in biological fluids. Please read the complete kit insert before performing this assay. The kit uses a monoclonal antibody to human IL-6 immobilized on a microtiter plate to bind the human IL-6 in the standards or sample. A recombinant human IL-6 Standard is provided in the kit. After a short incubation the excess sample or standard is washed out and a polyclonal antibody to human IL-6 is added. This antibody binds to the human IL-6 captured on the plate. After a short incubation the excess antibody is washed out and donkey anti-rabbit IgG conjugated to Horseradish peroxidase is added, which binds to the polyclonal human IL-6 antibody. Excess conjugate is washed out and substrate is added. After a short incubation, the enzyme reaction is stopped and the color generated is read at 450 nm. The measured optical density is directly proportional to the concentration of human IL-6 in either standards or samples. For further explanation of the principles and practices of immunoassays please see the excellent books by Chard<sup>1</sup> or Tijssen<sup>2</sup>.

## **Introduction**

Human Interleukin-6 (h IL-6) is a cytokine critical to the regulation of the immune and hematopoietic systems. It has been called interferon  $\beta_2$ , hybridoma/plasmacytoma growth factor, B-cell stimulatory factor 2, 26kDa inducible protein, hepatocyte stimulating factor, hematopoietic colony stimulating factor, monocyte granulocyte inducer type 2 and cytotoxic T-cell differentiation factor<sup>4,5,10</sup>.

A 212-amino acid glycoprotein, human IL-6 is expressed by cell types such as T-cells, mast cells, monocytes, macrophages, fibroblasts, endothelial cells, keratinocytes, and many tumor cell lines. It appears to take part in acute phase reactions and response to injury and inflammation<sup>4,5,9</sup>. Human IL-6 also stimulates the differentiation of B-cells for antibody production, promotes expansion of activated T-cells, expands hematopoietic cell production, and induces the expression of acute phase proteins<sup>5-10</sup>.

Levels of human IL-6 have been shown to increase rapidly in serum with sepsis and burn trauma, and in cerebrospinal fluid with acute viral or bacterial infection of the CNS<sup>9</sup>. Human IL-6 is implicated in conditions including autoimmune diseases and polyclonal B-cell abnormalities<sup>4,9</sup>.

## **Precautions**

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1. Stop Solution 2 is a 1 normal (1N) hydrochloric acid solution. This solution is caustic; care should be taken in use.
2. The activity of the Horseradish peroxidase conjugate is affected by nucleophiles such as azide, cyanide and hydroxylamine.
3. We test this kit's performance with a variety of samples, however it is possible that high levels of interfering substances may cause variation in assay results.
4. The human IL-6 Standard provided, Catalog No. 80-0625, should be handled with care because of the known and unknown effects of IL-6.
5. The human IL-6 Standard should be aliquoted and stored at or below -20 °C. Do not repeatedly freeze-thaw.

## Materials Supplied

1. **human IL-6 Microtiter Plate, One Plate of 96 Wells, Catalog No. 80-0318**  
A plate using break-apart strips coated with monoclonal antibody specific to human IL-6.
2. **human IL-6 Antibody , 11 mL, Catalog No. 80-0384**  
A yellow solution of rabbit polyclonal antibody to human IL-6.
3. **Assay Buffer 13, 60 mL, Catalog No. 80-1562**  
Tris buffered saline containing proteins and detergents.
4. **human IL-6 Conjugate, 11 mL, Catalog No. 80-0869**  
A blue solution of donkey anti-rabbit IgG conjugated to Horseradish peroxidase.
5. **Wash Buffer Concentrate, 105 mL, Catalog No. 80-1287**  
Tris buffered saline containing detergents.
6. **human IL-6 Standard, 2 each, Catalog No. 80-0625**  
Two vials containing 5,000 pg each of lyophilized recombinant human IL-6. **Avoid repeated freeze/thaw cycles.**
7. **TMB Substrate, 12 mL, Catalog No. 80-0350**  
A solution of 3,3',5,5' tetramethylbenzidine (TMB) and hydrogen peroxide. Ready to use. **Protect from prolonged exposure to light.**
8. **Stop Solution 2, 11 mL, Catalog No. 80-0377**  
A 1N solution of hydrochloric acid in water. Keep tightly capped. Caution: **Caustic.**
9. **human IL-6 Assay Layout Sheet, 1 each, Catalog No. 30-0096**
10. **Plate Sealer, 3 each, Catalog No. 30-0012**

## Storage

All components of this kit, **except the human IL-6 Standards**, are stable at 4 °C until the kit's expiration date. The Standards **must** be stored at or below -20 °C.

## Materials Needed but Not Supplied

1. Deionized or distilled water. No difference in assay results is seen with distilled water.
2. Precision pipets for volumes between 100 µL and 1,000 µL.
3. Disposable test tubes for dilution of samples and standards.
4. Repeater pipets for dispensing 100 µL.
5. Disposable beakers for diluting buffer concentrates.
6. Graduated cylinders.
7. A microplate shaker.
8. Adsorbent paper for blotting.
9. Microplate reader capable of reading at 450 nm, preferably with correction between 570 nm and 590 nm.
10. Graph paper for plotting the standard curve.

## **Sample Handling**

Assay Designs' TiterZyme® EIA is compatible with human IL-6 samples in culture fluids, serum, plasma, and urine. Samples diluted sufficiently into the assay buffer can be read directly from the standard curve. Please refer to the Sample Recovery recommendations on page 11 for details of suggested dilutions.

Samples containing a visible precipitate must be clarified prior to use in the assay. Do not use grossly hemolyzed or lipemic specimens. Samples in the majority of tissue culture media, including those containing fetal bovine serum, can be read in the assay, provided the standards have been diluted into the diluted non-conditioned tissue culture media instead of Assay Buffer 13. **Tissue culture media must be diluted at least 1:2 in Assay Buffer 13.** There will be a small change in binding associated with running the standards and samples in media. Users should only use standard curves generated in media or buffer to calculate concentrations of human IL-6 in the appropriate matrix.

Samples must be stored frozen to avoid loss of bioactive IL-6. If samples are to be run within 24 hours, they may be stored at 4°C. Otherwise, samples must be stored frozen at -70 °C to avoid loss of bioactive IL-6. Up to three freeze/thaw cycles of serum has been shown to have no effect on IL-6 levels. Nonetheless, excessive freeze/thaw cycles should be avoided. Prior to assay, frozen sera should be brought to room temperature slowly and gently mixed by hand. Do not thaw samples in a 37°C incubator. Do not vortex or sharply agitate samples.

## **Procedural Notes**

1. Do not mix components from different kit lots or use reagents beyond the kit expiration date.
2. Allow all reagents to warm to room temperature for at least 30 minutes before opening.
3. Standards can be made up in either glass or plastic tubes.
4. Pre-rinse the pipet tip with the reagent, use fresh pipet tips for each sample, standard and reagent.
5. Pipet standards and samples to the bottom of the wells.
6. Add the reagents to the side of the well to avoid contamination.
7. 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.
8. **Prior to addition of substrate, ensure that there is no residual wash buffer in the wells. Any remaining wash buffer may cause variation in assay results.**
9. **It is important that the matrix for the standards and samples be as similar as possible. Human IL-6 samples diluted with Assay Buffer 13 should be run with a standard curve diluted in the same buffer. Serum and plasma samples should be evaluated against a standard curve run in Assay Buffer 13, while Tissue Culture samples should be read against a standard curve diluted in the same complete but non-conditioned media. See Reagent Preparation, step #2.**

## **Reagent Preparation**

### **1. Wash Buffer**

Prepare the Wash Buffer by diluting 50 mL of the supplied concentrate with 950 mL of deionized water. This can be stored at room temperature until the kit expiration, or for 3 months, whichever is earlier.

### **2. human IL-6 Standards**

Reconstitute one vial of human IL-6 Standard with 500 µL of standard diluent (Assay Buffer 13 or Tissue Culture Media). Mix thoroughly without foaming.

Label seven 12x75mm glass tubes #1 through #7. Pipet 950 µL of standard diluent into tube #1. Pipet 500 µL of standard diluent into tubes #2 through #7. Add 50 µL of the 10,000 pg/mL Standard to tube #1. Vortex thoroughly. Add 500 µL of tube #1 to tube #2 and vortex thoroughly. Add 500 µL of tube #2 to #3 and vortex thoroughly. Continue this for tubes #4 through #7.

**The concentration of human IL-6 in tubes #1 through #7 will be 500, 250, 125, 62.5, 31.25, 15.63, and 7.81 pg/mL respectively. See human IL-6 Assay Layout for dilution details. STORE STANDARD AT -20 °C, avoid repeated freeze-thaws.**

## Assay Procedure

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

**Plates require shaking on a microplate shaker at 500 rpm.**

**All standards, controls and samples should be run in duplicate.**

1. Refer to the Assay Layout Sheet to determine the number of wells to be used and put any remaining wells with the desiccant back into the pouch and seal the ziploc. Store unused wells at 4 °C.
2. Pipet 100 µL of standard diluent (Assay Buffer 13 or Tissue Culture Media) into the S0 (0 pg/mL standard) wells.
3. Pipet 100 µL of Standards #1 through #7 into the appropriate wells.
4. Pipet 100 µL of the Samples into the appropriate wells.
5. Tap the plate gently to mix the contents, and seal with the plate sealer.
6. Seal the plate and incubate at room temperature, on a plate shaker for 1 hour at ~500rpm.
7. Empty the contents of the wells and wash by adding 400 µL of wash solution to every well. Repeat the wash 3 more times for a total of **4 washes**.
8. Pipet 100 µL of yellow human IL-6 Antibody into each well, except the Blank.
9. Seal the plate and incubate at room temperature, on a plate shaker for 1 hour at ~500rpm.
10. Empty the contents of the wells and wash by adding 400 µL of wash solution to every well. Repeat the wash 3 more times for a total of **4 washes**.
11. Add 100 µL of blue human IL-6 Conjugate to each well, except the Blank.
12. Seal the plate and incubate at room temperature on a plate shaker for 30 minutes at ~500rpm.
13. Empty the contents of the wells and wash by adding 400 µL of wash solution to every well. Repeat the wash 3 more times for a total of **4 washes**.
14. Pipet 100 µL of Substrate Solution into each well.
15. Incubate for 15 minutes on a plate shaker, at room temperature at ~500rpm.
16. Pipet 100 µL Stop Solution 2 to each well. This stops the reaction and the plate should be read immediately.
17. Blank the plate reader against the Blank wells, read the optical density at 450 nm, preferably with correction between 570 and 590 nm. If the plate reader is not able to be blanked against the Blank wells, manually subtract the mean optical density of the Blank wells from all the readings.

## Calculation of Results

Several options are available for the calculation of the concentration of human IL-6 in the samples. We recommend that the data be handled by an immunoassay software package utilizing a 4 parameter logistic curve fitting program. If data reduction software is not readily available, the concentration of human IL-6 can be calculated as follows:

1. Calculate the average net Optical Density (OD) bound for each standard and sample by subtracting the average Blank OD from the average OD for each standard and sample.

$$\text{Average Net OD} = \text{Average OD} - \text{Average Blank OD}$$

2. Using linear graph paper, plot the Average Net OD for each standard versus human IL-6 concentration in each standard. Approximate a straight line through the points. The concentration of human IL-6 in the unknowns can be determined by interpolation.

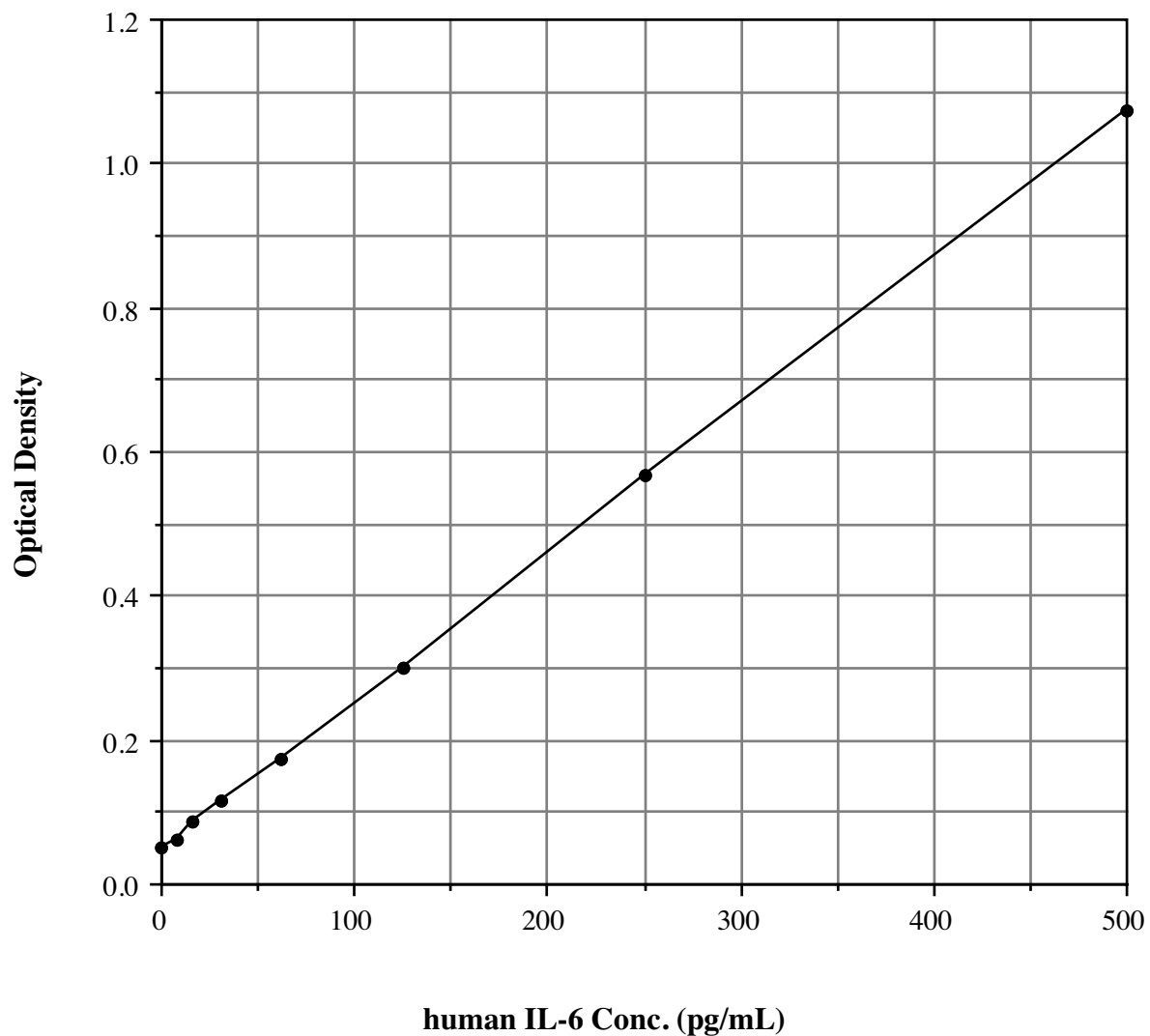
## Typical Results

The results shown below, from the regular assay procedure, are for illustration only and **should not** be used to calculate results from another assay.

<u>Sample</u>	<u>Average OD</u>	<u>Net OD</u>	<u>h IL-6 (pg/mL)</u>
Blank	0.055		
S0	0.107	0.052	<b>0</b>
S1	1.128	1.073	<b>500</b>
S2	0.622	0.567	<b>250</b>
S3	0.356	0.301	<b>125</b>
S4	0.230	0.175	<b>62.5</b>
S5	0.172	0.117	<b>31.25</b>
S6	0.140	0.085	<b>15.63</b>
S7	0.118	0.063	<b>7.81</b>
Unknown 1	0.356	0.301	<b>125.24</b>
Unknown 2	0.262	0.207	<b>78.62</b>

### Typical Standard Curve

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





## **Performance Characteristics**

The following parameters for this kit were determined using the guidelines listed in the National Committee for Clinical Laboratory Standards (NCCLS) Evaluation Protocols<sup>11</sup>.

### **Sensitivity**

Sensitivity was calculated by determining the average optical density bound for sixteen (16) wells run with 0 pg/mL Standard, and comparing to the average optical density for sixteen (16) wells run with Standard #7. The detection limit was determined as the concentration of human IL-6 measured at two (2) standard deviations from the 0 pg/mL Standard along the standard curve.

Mean OD for S0 = 0.047 ± 0.005 (9.7%)

Mean OD for Standard #7 = 0.060 ± 0.004 (6.0%)

Delta Optical Density = (7.81 - 0 pg/mL) = 0.060 - 0.047 = 0.013

2 SD's of 0 pg/mL Standard = 2 x 0.005 = 0.010

Sensitivity =  $\frac{0.010}{0.013}$  x 7.81 pg/mL = **6.01 pg/mL**

### **Linearity**

A sample containing 274.6 pg/mL mouse IL-6 was serially diluted 4 times 1:2 in the Assay Buffer 13 supplied in the kit and measured in the assay. The data was plotted graphically as actual human IL-6 concentration versus measured human IL-6 concentration.

The line obtained had a slope of 0.9211 with a correlation coefficient of 0.9917.

## Precision

Intra-assay precision was determined by taking samples containing low, medium and high concentrations of human IL-6 and running these samples multiple times (n=16) in the same assay. Inter-assay precision was determined by measuring three samples with low, medium and high concentrations of human IL-6 in multiple assays (n=9).

The precision numbers listed below represent the percent coefficient of variation for the concentrations of human IL-6 determined in these assays as calculated by a 4 parameter logistic curve fitting program.

	h IL-6 (pg/mL)	Intra-assay <u>% CV</u>	Inter-assay <u>% CV</u>
Low	109.8	3.8	
Medium	166.8	5.7	
High	250.7	6.4	
Low	95.2		14.4
Medium	186.6		12.6
High	256.8		10.3

## Cross Reactivities

The human IL-6 TiterZyme<sup>®</sup> EIA Kit is specific for bioactive human IL-6. The kit is unaffected by the presence of recombinant mouse IL-6 or recombinant human IL-1 $\alpha$ , IL-1 $\beta$ , IL-2, IL-3, IL-4, IL-7, IL-8, TNF- $\alpha$ , TNF- $\beta$ , GM-CSF and G-CSF.

## Sample Recoveries

Please refer to pages 4 and 5 for Sample Handling recommendations and Standard preparation.

Human IL-6 concentrations were measured in a variety of different samples including tissue culture media, human serum, EDTA plasma and urine. Human IL-6 was spiked into samples of these matrices that were diluted with the assay buffer and assayed in the kit. The following results were obtained:

<u>Sample</u>	<u>% Recovery</u> *	<u>Recommended Dilution</u> *
Tissue Culture Media	104.9	1:2
Human Serum	88.3	1:4
Human EDTA Plasma	100.1	1:8
Human Urine	91.9	1:4

\* See Sample Handling instructions on page 4 for details.

## References

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5. Kishimoto, The biology of Interleukin-6. *Blood* 74:1 (1989).
6. T. Kishimoto and T. Hirano, Molecular regulation of  $\beta$  lymphocyte response. *Annu. Rev. Immunol.* 6:485 (1988).
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10. Wong, G. and Clark, S. Multiple actions of Interleukin-6 within a cytokine network. *Immunol. Today* 9:137 (1988).
11. National Committee for Clinical Laboratory Standards Evaluation Protocols, SC1, (1989) Villanova, PA: NCCLS.

## **LIMITED WARRANTY**

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**Material Safety Data Sheet (MSDS) available on our website or by fax.**

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