



Rat IL-6 ELISA

670 010 096 1 x 96 tests
670 010 192 2 x 96 tests

INTENDED USE

The rat IL-6 ELISA is an enzyme-linked immunosorbent assay for quantitative detection of rat Interleukin-6 in cell culture supernatants, murine serum, plasma or other body fluids. **The rat IL-6 ELISA is for research use only. Not for use in diagnostic or 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 (3, 7).

IL-6 is usually not produced constitutively by normal cells, but its expression is readily induced by a variety of cytokines (6), lipopolysaccharide (5) or viral infections (1).

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 (4)

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 (2). 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.

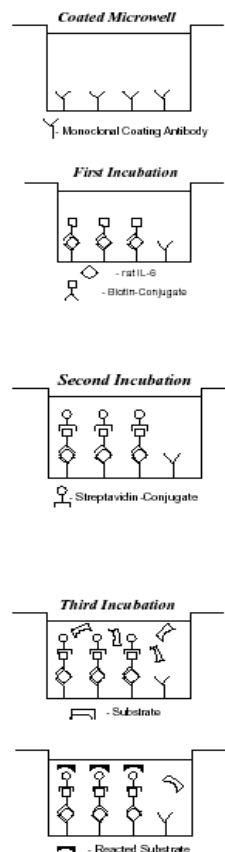
PRINCIPLES OF THE METHOD

An anti-rat IL-6 monoclonal coating antibody is adsorbed onto microwells.

rat IL-6 present in the sample or standard binds to antibodies adsorbed to the microwells; a biotin-conjugated monoclonal anti-rat IL-6 antibody is added and binds to rat IL-6 captured by the first antibody.

Following incubation unbound biotin conjugated anti-rat IL-6 is removed during a wash step. Streptavidin-HRP is added and binds to the biotin conjugated anti-rat IL-6. Following incubation unbound Streptavidin-HRP is removed during a wash step, and substrate solution reactive with HRP is added to the wells.

A coloured product is formed in proportion to the amount of rat IL-6 present in the sample. The reaction is terminated by addition of acid and absorbance is measured at 450nm. A standard curve is prepared from seven rat IL-6 standard dilutions and rat rat IL-6 sample concentration determined.



REAGENTS PROVIDED

REAGENTS (store at 2-8°C)	QUANTITY 1 plate	QUANTITY 2 plates	RECONSTITUTION
96-wells precoated microtiter plate	1	2	Ready-to-use
Plate covers	4	8	
rIL-6 Standard: 4000 pg/ml	2 vials	4 vials	Reconstitute with the volume of distilled water indicated on the vial.
Biotin conjugate anti-rat IL-6 monoclonal antibody ^{1,2}	1 vial	2 vials	(100 µl) Dilute 100 times in Assay buffer
Streptavidin-HRP ^{1,2}	1 vial	2 vials	(150 µl) Make a 1/100 dilution with Assay Buffer before use.
Assay Buffer Concentrate	1 vial	2 vials	(5 ml) 20X concentrate. Dilute in distilled water
Wash Buffer Concentrate	1 bottle	2 bottles	(50 ml) 20X concentrate. Dilute in distilled water
Substrate Solution	1 vial	2 vials	(15ml) Ready-to-use.
Stop Solution (1M Phosphoric acid)	1 vial	2 vials	(12 ml) Ready-to-use
Blue Dye	1 vial	2 vials	(0.4 ml) Make a 1/250 dilution in the appropriate diluent
Red Dye	1 vial	2 vials	(0.4 ml) Make a 1/250 dilution in the Assay Buffer
Green Dye	1 vial	2 vials	(0.4 ml) Make a 1/100 dilution in the Assay Buffer

1) reagents contain preservative

2) It is recommended to spin vial in microcentrifuge before use to collect reagent at the bottom.

MATERIALS REQUIRED BUT NOT PROVIDED

- 5 ml and 10 ml graduated pipettes
- 10 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
- 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 research use only and are not for use in diagnostic or therapeutic 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.
- Avoid contact of substrate solutions with oxidizing agents and metal.
- 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 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.
- Liquid wastes not containing acid and neutralized waste may be mixed with sodium hypochlorite in volumes such that the final mixture contains 1.0 % sodium hypochlorite. Allow 30 minutes for effective decontamination. Liquid waste containing acid must be neutralized prior to the addition of sodium hypochlorite.

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, murine 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.

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

Clinical samples should be kept at 2° to 8°C and separated rapidly before storing at -20°C to avoid loss of bioactive rat IL-6. If samples are to be run within 24 hours, they may be stored at 2° to 8°C. Avoid repeated freeze-thaw cycles.

PREPARATION OF REAGENTS

1. Wash Buffer

If crystals have formed in the Wash Buffer Concentrate, warm it gently until they have completely dissolved.

Pour entire contents (50 ml) of the **Wash Buffer Concentrate** into a clean 1,000 ml graduated cylinder. Bring final volume to 1,000 ml with glass-distilled or deionized water. Mix gently to avoid foaming. The pH of the final solution should adjust to 7.4.

Transfer to a clean wash bottle and store at 2° to 25°C. Please note that the Wash Buffer is stable for 30 days. Wash Buffer may be prepared as needed according to the following table:

Number of Strips	Wash Buffer Concentrate (ml)	Distilled Water (ml)
1 - 6	25	475
1 - 12	50	950

2. Assay Buffer

Mix the contents of the bottle well. Add contents of **Assay Buffer Concentrate** (5.0 ml) to 95 ml distilled or deionized water and mix gently to avoid foaming. Store at 2° to 8°C. Please note that the Assay Buffer is stable for 30 days. Assay Buffer may be prepared as needed according to the following table.

Number of Strips	Assay Buffer Concentrate (ml)	Distilled Water (ml)
1 - 6	2.5	47.5
1 - 12	5.0	95.0

3. Preparation of Biotin-Conjugate

Make a 1:100 dilution with **Assay Buffer** in a clean plastic tube as needed according to the following table:

Number of Strips	Biotin-Conjugate (ml)	Assay Buffer (ml)
1 - 6	0.03	2.97
1 - 12	0.06	5.94

4. Preparation of rIL-6 Standard

Reconstitute rIL-6 Standard by addition of distilled water. Reconstitutions volume is stated on the label of the standard vial. Mix gently to ensure complete solubilization. Store reconstituted Standard promptly at –20°C. Discard after one week.

5. Preparation of Streptavidin-HRP

Make a 1:100 dilution of the concentrated **Streptavidin-HRP** solution in **Assay Buffer** as needed according to the following table:

Number of Strips	Streptavidin-HRP (ml)	Assay Buffer (ml)
1 - 6	0.06	6
1 - 12	0.12	12

6. Addition of Colour-giving Dyes

In order to help our customers to avoid any mistakes in pipetting, DIACLONE now offers a new tool that helps to monitor the addition of even very small volumes of a solution to the reaction well by giving distinctive colours to each step of the ELISA procedure.

This procedure is optional, does not in any way interfere with the test results, and is designed to help the customer with the performance of the test, but can also be omitted, just following the instruction booklet.

Alternatively, the dye solutions from the stocks provided (**Blue-Dye, Green-Dye, Red-Dye**) can be added to the reagents according to the following guidelines:

A. Diluent:

Before sample dilution add the **Blue-Dye** at a dilution of 1:250 (see table below) to the appropriate diluent (1x) according to the test protocol. After addition of **Blue-Dye**, proceed according to the instruction booklet.

5 ml Diluent	20 µl Blue-Dye
12 ml Diluent	48 µl Blue-Dye

B. Biotin-Conjugate:

Before dilution of the concentrated conjugate, add the **Green-Dye** at a dilution of 1:100 (see table below) to the Assay Buffer (1x) used for the final conjugate dilution. Proceed after addition of **Green-Dye** according to the instruction booklet, preparation of Biotin-conjugate.

3 ml Assay Buffer	30 µl Green-Dye
6 ml Assay Buffer	60 µl Green-Dye

C. Streptavidin-HRP:

Before dilution of the concentrated Streptavidin-HRP, add the **Red-Dye** at a dilution of 1:250 (see table below) to the Assay Buffer (1x) used for the final Streptavidin-HRP dilution. Proceed after addition of **Red-Dye** according to the instruction booklet, preparation of Streptavidin-HRP.

6 ml Assay Buffer	24 µl Red-Dye
12 ml Assay Buffer	48 µl Red-Dye

TEST PROTOCOL

- a. Prepare reagents immediately before use and mix them thoroughly without foaming.
- 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 optional control sample should be assayed in duplicate. Remove extra **Microwell Strips coated with Monoclonal Antibody** to rat IL-6 from holder and store in foil bag with the dessicant provided at 2°-8°C sealed tightly.
- c. Wash the microwell strips twice with approximately 300 µl **Wash Buffer** per well with thorough aspiration of microwell contents between washes. Take care not to scratch the surface of the microwells. After the last wash, empty wells and tap microwell strips on absorbent pad or paper towel to remove excess Wash Buffer. Use the microwell strips immediately after washing or place upside down on a wet absorbent paper for not longer than 15 minutes. Do not allow wells to dry.
- d. Add 100 µl of **Assay Buffer** in duplicate to all standard wells. Prepare standard dilutions by pipetting 100 µl of reconstituted (Refer to preparation of reagents) **rat IL-6 Standard**, in duplicate, into wells A1 and A2. Mix the contents of wells A1 and A2 by repeated aspiration and ejection, and transfer 100 µl to well B1 and B2, respectively. Take care not to scratch the inner surface of the microwells. Continue this procedure five times, creating two rows of rat IL-6 standard dilutions ranging from 2000 to 31,3 pg/ml. Discard 100 µl of the contents from the last microwells (G1, G2) used.

Figure 1. Preparation of rat IL-6 standard dilutions:

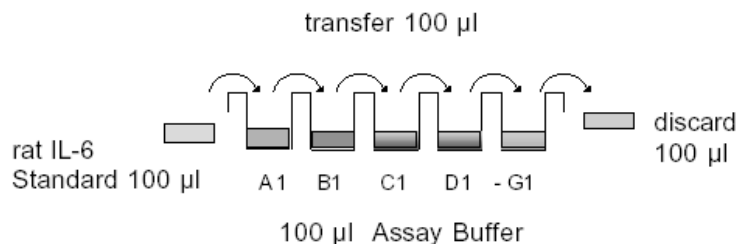


Figure 2. Diagram depicting an example of the arrangement of blanks, standards and samples in the microwell strips:

	Standard Concentrations pg/mL		Sample wells									
	1	2	3	4	5	6	7	8	9	10	11	12
A	2000	2000										
B	1000	1000										
C	500	500										
D	250	250										
E	125	125										
F	62.5	62.5										
G	31.3	31.3										
H	Blank	Blank										

- e. Add 100 µl of **Assay Buffer**, in duplicate, to the blank wells.
- f. Add 50 µl of **Assay Buffer** to the sample wells.
- g. Add 50 µl of each **Sample**, in duplicate, to the designated wells.
- h. Prepare **Biotin-Conjugate**. (Refer to preparation of reagents)
- i. Add 50 µl of diluted **Biotin-Conjugate** to all wells, including the blank wells.
- j. Cover with a **Plate Cover** and incubate at room temperature (18° to 25°C) for 2 hours on a microplate shaker set at 200 rpm.
- k. Remove Plate Cover and empty wells. Wash microwell strips 3 times according to point c. of the test protocol. Proceed immediately to the next step.
- l. Prepare **Streptavidin-HRP**. (Refer to preparation of reagents)
- m. Add 100 µl of diluted **Streptavidin-HRP** to all wells, including the blank wells.
- n. Cover with a Plate Cover and incubate at room temperature (18° to 25°C) for 1 hour on a microplate shaker at 200 rpm.
- o. Remove Plate Cover and empty wells. Wash microwell strips 3 times according to point c. of the test protocol. Proceed immediately to the next step.
- p. Pipette 100 µl of **TMB Substrate Solution** to all wells, including the blank wells.
- q. Incubate the microwell strips at room temperature (18° to 25°C) for about **15 minutes** on a microplate shaker at 200 rpm. Avoid direct exposure to intense light. The point at which the substrate reaction need to be stopped is often determined by the ELISA reader being used. 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. The substrate reaction must be stopped before positive wells are no longer properly recordable.**

- r. Stop the enzyme reaction by quickly pipetting 100 µl of **Stop Solution** into each well, including the blank wells. It is important that the Stop Solution is spread quickly and uniformly throughout the microwells to completely inactivate the enzyme. Results must be read immediately after the Stop Solution is added or within one hour if the microwell strips are stored at 2 - 8°C in the dark.
- s. 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 and the rat IL-6 standards.

Note: In case of incubation without shaking the obtained O.D. values may be lower than indicated below. Nevertheless the results are still valid.

CALCULATION OF RESULTS

- Calculate the average absorbance values for each set of duplicate standards and samples. Duplicates should be within 20 per cent of the mean.
- Create a standard curve by plotting the mean absorbance for each standard concentration on the ordinate against the rat IL-6 concentration on the abscissa. Draw a best fit curve through the points of the graph.
- To determine the concentration of circulating rat IL-6 for each sample, first find the mean absorbance 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 rat IL-6 concentration.

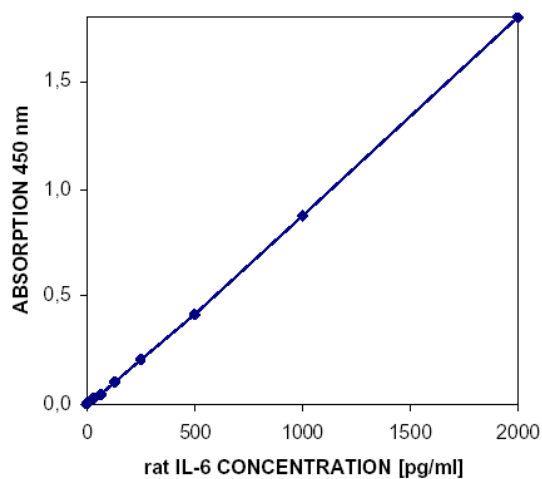
If samples have been diluted according to the instructions given in this manual (e.g. cell culture supernatants), the concentration read from the standard curve must be multiplied by the respective dilution factor.

Note: Calculation of samples with an O.D. exceeding 2.0 may result in incorrect, low rat IL-6 levels. Such samples should be re-analyzed at higher dilution rate in order to precisely quantitate the actual rat IL-6 level.

It is suggested that each testing facility establishes a control sample of known rat IL-6 concentration and runs this additional control with each assay. If the values obtained are not within the expected range of this control, the assay results may be invalid.

- 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 rat IL-6 ELISA. Rat IL-6 was diluted in serial two-fold steps in Assay Buffer, symbols represent the mean of three parallel titrations. 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 rat IL-6 ELISA

Measuring wavelength: 450 nm

Reference wavelength: 620 nm

Standard	Rat IL-6 Concentration (pg/ml)	O.D. (450 nm)	O.D. Mean	C.V. (%)
1	2000	1.863	1.839	1.8
	2000	1.815		
2	1000	0.896	0.910	2.1
	1000	0.923		
3	500	0.459	0.457	0.6
	500	0.455		
4	250	0.243	0.247	2.0
	250	0.250		
5	125	0.139	0.139	0.5
	125	0.138		
6	62.5	0.082	0.083	0.9
	62.5	0.083		
7	31.3	0.064	0.064	0.0
	31.3	0.064		
Blank		0.038	0.038	
		0.037		

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 Wash Buffer, fill with Wash 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 of rat IL-6 defined as the analyte concentration resulting in an absorption significantly higher than that of the dilution medium (mean plus three standard deviations) was determined to be 19 pg/ml (mean of 6 independent assays).

Reproducibility

a. Intra-assay

Reproducibility within the assay was evaluated in independent experiments. The overall intra-assay coefficient of variation has been calculated to be <5%.

b. Inter-assay

Assay to assay reproducibility within one laboratory was evaluated in independent experiments by two technicians. The overall inter-assay coefficient of variation has been calculated to be <10%.

Dilution Parallelism

Rat serum spiked with different levels of rat IL-6 was assayed at four serial twofold dilutions with 4 replicates each. Experiments showed an overall mean recovery of 97 %.

Sample Stability

a. Freeze-Thaw Stability

Aliquots of spiked serum were stored frozen at -20°C and thawed up to 5 times, and rat IL-6 levels determined. There was no significant loss of rat IL-6 by freezing and thawing up to 5 times.

b. Storage Stability

Aliquots of spiked serum were stored at -20°C, 2-8°C, room temperature (RT) and at 37°C, and the rat IL-6 level determined after 24 h. There was no significant loss of rat IL-6 immunoreactivity during storage at -20°C, 4°C and room temperature. Storage at 37°C gave rise to loss of rat IL-6 immunoreactivity.

Specificity

The interference of circulating factors of the immune systems was evaluated by spiking these proteins at physiologically relevant concentrations into a rat IL-6 positive serum. There was no detectable cross reactivity.

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REAGENT PREPARATION SUMMARY

A. Wash Buffer Add **Wash Buffer Concentrate** 20 x (50 ml) to 950 ml distilled water

B. Assay Buffer	Number of Strips	Assay Buffer Concentrate (ml)	Distilled Water (ml)
	1 - 6	2.5	47.5
	1 - 12	5.0	95.0

C. Biotin-Conjugate Make a 1:100 dilution according to the table.

	Number of Strips	Biotin-Conjugate (ml)	Assay Buffer (ml)
	1 - 6	0.03	2.97
	1 - 12	0.06	5.94

D. Standard Reconstitute rat mL-6 Standard by addition of distilled water. Reconstitutions volume is stated on the label of the standard vial.

E. Streptavidin-HRP	Number of Strips	Streptavidin-HRP (ml)	Assay Buffer (ml)
	1 - 6	0.06	6.0
	1 - 12	0.12	12.0

TEST PROTOCOL SUMMARY

- Wash microwell strips twice with **Wash Buffer**
- Add 100 µl **Assay Buffer**, in duplicate, to all standard wells
- Pipette 100 µl diluted **rat IL-6 Standard** into the first wells and create standard dilutions ranging from 2000 to 31.3 pg/ml by transferring 100 µl from well to well. Discard 100 µl from the last wells.
- Add 100 µl **Assay Buffer**, in duplicate, to the blank wells
- Add 50 µl **Assay Buffer**, in duplicate, to the sample wells
- Add 50 µl **Sample**, in duplicate, to designated wells
- Prepare **Biotin-Conjugate**
- Add 50 µl of diluted **Biotin-Conjugate** to all wells
- Cover microwell strips and incubate 2 hours at room temperature (18° to 25°C) on microplate shaker
- Prepare **Streptavidin-HRP**
- Empty and wash microwell strips 3 times with **Wash Buffer**
- Add 100 µl of diluted **Streptavidin-HRP** to all wells
- Cover microwell strips and incubate 1 hour at room temperature (18° to 25°C) on microplate shaker
- Empty and wash microwell strips 3 times with **Wash Buffer**
- Add 100 µl of **TMB Substrate Solution** to all wells including blank wells
- Incubate the microwell strips for 15 minutes at room temperature (18°to 25°C) on microplate shaker
- Add 100 µl **Stop Solution** to all wells including blank wells
- Blank microwell reader and measure colour intensity at 450 nm