Rat IL-6 ELISA Kit

Instructions for use

Catalogue numbers: 1x96 tests: 670.010.096

For research use only

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Table of Contents

Rat IL-6 ELISA KIT

1. Intended use

The Rat IL-6 ELISA kit is a solid phase sandwich ELISA for the *in-vitro* qualitative and quantitative determination of rIL-6 in supernatants, buffered solutions or serum samples.

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

2. Introduction

2.1. 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.

2.2. Principle of the method

A capture Antibody highly specific for rIL-6 has been coated to the wells of the microtiter strip plate provided during manufacture. Binding of rIL-6 samples and known standards to the capture antibodies and subsequent binding of the Biotinylated anti-rIL-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 rIL-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 rIL-6 in any sample tested.

3. Reagents provided and reconstitution

Reagents (Store@2-8°C)	**Quantity 1x96-well kit** Cat no. 670.010.096	**Reconstitution**
Anti-rat IL-6 Coated Plate	1	Ready to use (96 well strip pre-coated plate)
Plastic plate covers	2	n/a
Rat IL-6 Standard: 4000 pg/ml	2	Reconstitute as directed on the vial (see Assay preparation, section 8)
Assay Buffer	1 (5ml)	20x concentrate, dilute in distilled water (see Assay preparation, section 8)
Biotin Conjugate Anti-rat IL-6	1 (70µl)	Dilute in Assay Buffer 1X (see Assay preparation, section 8)
Streptavidin-HRP	1 (150µl)	Dilute in Assay Buffer 1X (see Assay preparation, section 8)
Wash Buffer	1 (50ml)	20x concentrate dilute in distilled water (see Assay preparation, section 8)
Substrate Solution	1 (15ml)	Ready to use
Stop Solution	1 (15ml)	Ready to use

4. Materials required but not provided

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

5. Storage Instructions

Store kit reagents between 2 and 8°C. Immediately after 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.

Wash Buffer 1X: Once prepared, store at 2-8°C for up to 1 week.

Assay Buffer 1X: Once prepared, store at 2-8°C for up to 1 week.

Reconstituted Standard: Once prepared use immediately and do not store.

Diluted Biotin conjugate Anti-rat IL-6: Once prepared use immediately and do not store.

Diluted Streptavidin-HRP: Once prepared use immediately and do not store.

6. 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.

Storage: If not analysed shortly after collection, samples should be aliquoted (250-500µl) to avoid repeated freeze-thaw cycles and stored frozen at –70°C. Avoid multiple freeze-thaw cycles of frozen specimens.

Recommendation: Do not thaw by heating at 37°C or 56°C. Thaw at room 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.

7. 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.
- Laboratory gloves should be worn at all times.
- Avoid any skin contact with Stop and Substrate Solutions. 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 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 Stop and Substrate Solutions, 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 Substrate Solution is light sensitive. Avoid prolonged exposure to light. Also, avoid contact of the Substrate Solution with metal to prevent colour development. Warning Substrate solution 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 Substrate Solution has been contaminated and must be discarded. Read absorbances 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 Substrate solution within 15 min of the washing of the microtiter plate.

8. Assay Preparation

Bring all reagents to room temperature before use

8.1. 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 should be tested **in duplicate**. Remove sufficient microwell strips for testing from the 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)

	Standards		Sample Wells									
	1	2	3	4	5	6	7	8	9	10	11	12
A	Std 1	Std 1										
B	Std 2	Std 2										
C	Std 3	Std 3										
D	Std 4	Std 4										
E	Std 5	Std 5										
F	Std 6	Std 6										
G	zero	zero										
H												

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

8.2. Preparation of Wash Buffer

If crystals have formed in the concentrate Wash Buffer, warm it gently until complete dissolution.

Dilute the (20X) concentrate Wash Buffer 20 fold with distilled water to give a 1X working solution.
Pour entire contents (50 ml) of the concentrate Wash Buffer 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. Transfer to a clean wash bottle.

8.3. Preparation of Assay Buffer 1X

If crystals have formed in the concentrate Assay Buffer, warm it gently until complete dissolution.

Dilute the (20X) concentrate Assay Buffer 20 fold with distilled water to give a 1X working solution.
Add contents of concentrate Assay Buffer (5 ml) to 95 ml of distilled water. Mix gently to avoid foaming.

8.4. Preparation of Standard

Standard vials must be reconstituted with the volume of distilled water shown on the vial immediately prior to use. This reconstitution gives a stock solution of 4000 pg/ml of rat IL-6. Mix the reconstituted standard gently by inversion only. Serial dilutions of the standard are made directly in the assay plate to provide the concentration range from 2000 to 62.5 pg/ml. A fresh standard curve should be produced for each new assay.

- Add 100 µl of Assay Buffer 1X to all standard and blank wells.
- Immediately after reconstitution add 100µl of the reconstituted standard to wells A1 and A2, which provides the highest concentration standard at 2000 pg/ml. Mix the well contents by repeated aspirations and ejections taking care not to scratch the inner surface of the wells.
- Transfer 100µl 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 100µl from wells B1 and B2 through to wells F1 and F2 providing a serial diluted standard curve ranging from 2000 pg/ml to 62.5 pg/ml.
- Discard 100µl from the final wells of the standard curve (F1 and F2).

Alternatively these dilutions can be performed in separate clean tubes and immediately transferred into the relevant wells.

8.5. Preparation of Biotin Conjugate Anti-rat IL-6

It is recommended this reagent is prepared immediately before use. Dilute the Biotin Conjugate Anti-rat IL-6 with the Assay buffer 1X in an appropriate clean glass vial using volumes appropriate to the number of required wells. Please see example volumes below:

Number of strips	Biotin Conjugate (µl)	Assay Buffer 1X (ml)
1 - 6	30	2.97
1 - 12	60	5.94

8.6. Preparation of Streptavidin-HRP

It is recommended this reagent is prepared immediately before use. Dilute the Streptavidin-HRP with the Assay buffer 1X in an appropriate clean glass vial using volumes appropriate to the number of required wells. Please see example volumes below:

Number of strips	Streptavidin-HRP (µl)	Streptavidin-HRP Diluent (ml)
1 - 6	60	5.94
1 - 12	120	11.88

9. Method

We strongly recommend that every vial is mixed thoroughly without foaming prior to use.

Prepare all reagents as shown in section 8.
Note: final preparation of Biotin Conjugate (section 8.5) and Streptavidin-HRP (section 8.6) should occur immediately before use.

Assay Step		Details
1.	Wash	Remove the pre-coated plate from the sealed pouch, removed any un-needed strips and wash the plate as follows: a) Dispense 0.3 ml of **1x washing solution** into each well b) Aspirate the contents of each well c) Repeat step a and b
2.	Addition	**Prepare Standard curve** as shown in section 8.4
3.	Addition	Add 100 µl of **Assay Buffer 1X** in duplicate to the blank wells
4.	Addition	Add 50 µl of **Assay Buffer 1X** to the sample wells
5.	Addition	Add 50 µl of each **sample** in duplicate to the designated wells
6.	Addition	Add 50 µl of diluted **Biotin Conjugate** to all wells including blanks
7.	Incubation	Cover with a plastic plate cover and incubate at room temperature (18 to 25°C) for **2 hours** on a rotator set at 400rpm if available
8.	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 five times
9.	Addition	Add 100 µl of diluted **Streptavidin-HRP** solution into all wells
10.	Incubation	Cover with a plastic plate cover and incubate at room temperature (18 to 25°C) for **1 hour** on a rotator set at 400rpm if available
11.	Wash	Repeat wash step 8.
12.	Addition	Add 100 µl of ready-to-use **Substrate Solution** into all wells
13.	Incubation	Incubate for **10-20 minutes*** at room temperature. Avoid direct exposure to light by wrapping the plate in aluminium foil
14.	Addition	Add 100 µl of **Stop Solution** 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 TMB substrate 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.*

10. Data Analysis

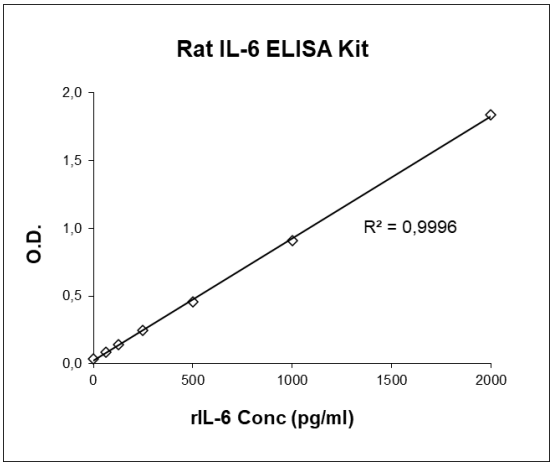
Calculate the average absorbance values for each set of duplicate standards and samples. Ideally duplicates should be within 20% of the mean.

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

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

Example Rat IL-6 Standard curve

Standard	rIL-6 Conc (pg/ml)	OD (450nm) mean	CV (%)
1	2000	1.839	1.8
2	1000	0.910	2.1
3	500	0.457	0.6
4	250	0.247	2.1
5	125	0.139	0.5
6	62.5	0.083	0.9
zero	0	0.038	-



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.

For samples human serum or plasmas which have been diluted 1:2 according to the protocol, the calculated concentration should be multiplied by the dilution factor (x2).

11. 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 Buffer 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 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.

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.**

12. Performance Characteristics

12.1. 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 two standard deviations) was determined to be 12 pg/ml (mean of 6 independent assays).

12.2. 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.

12.3. Precision

Intra-assay
Reproducibility within the assay was evaluated in 3 independent experiments. Each assay was carried out with 6 replicates of rat serum samples containing different concentrations of rat IL-6. Two standard curves were run on each plate. **The overall intra-assay coefficient of variation has been calculated to be <5%.**

Inter-assay
Assay to assay reproducibility within one laboratory was evaluated in 3 independent experiments. Each assay was carried out with 6 replicates of rat serum samples containing different concentrations of rat IL-6. Two standard curve were run on each plate. **The overall inter-assay coefficient of variation has been calculated to be <10%.**

12.4. Dilution Parallelism

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

12.5. Stability

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

Freeze-thaw Stability
Aliquots of spiked serum samples were stored at –20°C and thawed 5 times and the rat IL-6 level determined. There was no significant loss of rat IL-6 immunoreactivity detected by freezing and thawing.

13. Bibliography

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14. Assay Summary

Total procedure length: 3h10min

Add Samples, diluted Standards and diluted Biotin Conjugate

↓

Incubate 2 hours at room temperature

↓

Wash 6 times

↓

Add 100µl of diluted Streptavidin-HRP

↓

Incubate 1 hour at room temperature

↓

Wash 6 times

↓

Add 100µl of Substrate Solution
Protect from light. Let the color develop for 10-20 min.

↓

Add 100µl of Stop Solution

↓

Read Absorbance at 450 nm

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