Rat IL-10 ELISA Kit

Instructions for use

Catalogue numbers: 1x96 tests: 670.070.096

For research use only

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Rat IL-10 ELISA KIT

1. Intended use

The Diaclone Rat IL-10 ELISA kit is a solid phase sandwich ELISA for the *in-vitro* qualitative and quantitative determination of rIL-10.

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

2. Introduction

2.1. Summary

Interleukin-10 is a pleiotropic cytokine playing an important role as a regulator of lymphoid and myeloid cell function. Due to the ability of IL-10 to block cytokine synthesis and several accessory cell functions of macrophages, T-cells and NK cells. In addition, IL-10 participates in regulating proliferation and differentiation of B-cells, mast cells and thymocytes.
The murine IL-10 exhibits strong DNA and amino acid sequence homology to the human IL-10 and an open reading frame in the Epstein-Barr virus genome, BCRF1 which shares many of the cellular cytokine's biological activities and may therefore play a role in the host-virus interaction.

2.2. Principle of the method

A capture Antibody highly specific for rIL-10 has been coated to the wells of the microtiter strip plate provided during manufacture. Binding of rIL-10 samples and known standards to the capture antibodies and subsequent binding of the Biotin-Conjugate anti-rIL-10 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-10 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-10 in any sample tested.

3. Reagents provided and reconstitution

Reagents (Store@2-8°C)	**Quantity 1x96-well kit** Cat no. 670.070.096	**Reconstitution**
Anti-rat IL-10 Coated Plate	1	Ready to use (96 well strip pre-coated plate)
Plastic plate covers	2	n/a
Rat IL-10 Standard: 2000 pg/ml	2	Reconstitute as directed on the vial (see Assay preparation, section 8)
Sample Diluent	1 (12ml)	Ready to use
Assay Buffer	1 (5ml)	20x concentrate dilute in distilled water (see Assay preparation, section 8)
Biotin-Conjugate Anti-rat IL-10	1 (70µl)	Dilute in Assay Diluent 1X (see Assay preparation, section 8)
Streptavidin-HRP	1 (150µl)	Dilute in Assay Diluent 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-10: 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 supernatant and serum were tested with this assay. Other body fluids might be suitable for use in the assay. Remove serum from the clot as soon as possible after clotting.

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

Samples should be aliquoted and must be stored frozen at -20°C to avoid loss of bioactive rat IL-10. If samples are to be run within 24 hours, they may be stored at 2° to 8°C.
Avoid repeated freeze-thaw cycles. Prior to assay, the frozen sample should be brought to room temperature slowly and mixed gently.

Do not thaw samples in a 37°C water bath. Do not vortex or sharply agitate samples.
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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 TMB Substrate solution is light sensitive. Avoid prolonged exposure to light. Also, avoid contact of the TMB Substrate solution with metal to prevent colour development. Warning TMB Substrate 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 1X

Pour entire contents (50 ml) of the Wash Buffer Concentrate (20x) into a clean 1000 ml graduated cylinder. Bring to final volume of 1000 ml with glass-distilled or deionized water.
Mix gently to avoid foaming.
Transfer to a clean wash bottle and store at 2° to 25°C.
Wash Buffer (1x) may also be prepared as needed according to the following table:

Number of Strips	Wash Buffer concentrate 20X (ml)	Distilled water (ml)
1 - 6	25	475
1 - 12	50	950

8.3. Preparation of Assay Buffer 1X

Pour the entire contents (5 ml) of the Assay Buffer Concentrate (20x) into a clean 100 ml graduated cylinder. Bring to final volume of 100 ml with distilled water. Mix gently to avoid foaming.
Store at 2° to 8°C.
Assay Buffer (1x) may also be prepared as needed according to the following table:

Number of Strips	Standard Diluent concentrate (ml)	Distilled water (ml)
1 - 6	2.5	47.5
1 - 12	5.0	95.0

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 2000 pg/ml of rat IL-10. 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 1000 to 31.2 pg/ml. A fresh standard curve should be produced for each new assay.

- Add 100µl of Sample Diluent to the standard wells A1 and A2 to F1 and F2.
- Immediately after reconstitution, add 100µl of the reconstituted standard to wells A1 and A2, which provides the highest concentration standard at 1000 pg/ml.
- 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 1000 pg/ml to 31.2 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 Samples

Rat samples will be diluted 1:2 in Sample Diluent directly into wells.

8.6. Preparation of Biotin-Conjugate Anti-rat IL-10

Please note that the Biotin-Conjugate should be used within 30 minutes after dilution.
Make a 1:100 dilution of the concentrated Biotin-Conjugate solution with Assay buffer (1x) in a clean plastic tube as needed according to the following table:

Number of strips	Biotin-Conjugate (ml)	Assay Diluent 1X (ml)
1 - 6	0.03	2.97
1 - 12	0.06	5.94

8.7. Preparation of Streptavidin-HRP

Please note that the Streptavidin-HRP should be used within 30 minutes after dilution.
Make a 1:100 dilution of the concentrated Streptavidin-HRP solution with Assay Buffer (1x) in a clean plastic tube as needed according to the following table:

Number of Strips	Streptavidin-HRP (ml)	Assay Diluent 1X (ml)
1 - 6	0.06	5.94
1 - 12	0.12	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 8.6) and Streptavidin-HRP (section 8.7) should occur immediately before use.

Assay Step		Details
1.	Wash	a) Dispense 0.4 ml of **1x washing solution** into each well b) Aspirate the contents of each well c) Repeat steps a and b Do not allow wells to dry before use
2.	Addition	**Prepare Standard curve** as shown in section 8.4 and add 100µl of **Sample Diluent** to blank well (G1 and G2)
3.	Addition	Add 50µl of **Sample diluent** to all sample wells
4.	Addition	Add 50µl of **Sample** in duplicate to appropriate number of wells
5.	Addition	Add 50µl of diluted **biotin-conjugate** to all wells
6.	Incubation	Cover with a plastic plate cover and incubate at room temperature (18 to 25°C) for **2 hour(s)** if available on a microplate shaker set at 200 rpm
7.	Wash	Remove the cover and wash the plate as follows: a) Aspirate the liquid from each well b) Dispense 0.4 ml of **1x washing solution** into each well c) Aspirate the contents of each well d) Repeat step b and c another two times
8.	Addition	Add 100µl of diluted **Streptavidin-HRP** solution into all wells
9.	Incubation	Cover with a plastic plate cover and incubate at room temperature (18 to 25°C) for **1 hour** if available on a microplate shaker set at 200 rpm
10.	Wash	Repeat wash step 8.
11.	Addition	Add 100µl of ready-to-use **Substrate Solution** into all wells
12.	Incubation	Incubate in the dark for **10 minutes*** at room temperature. Avoid direct exposure to light by wrapping the plate in aluminium foil.
13.	Addition	Add 100µl of **Stop Solution** into all wells
Read the absorbance value of each well (immediately after step 14.) 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.*

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

10. Data Analysis

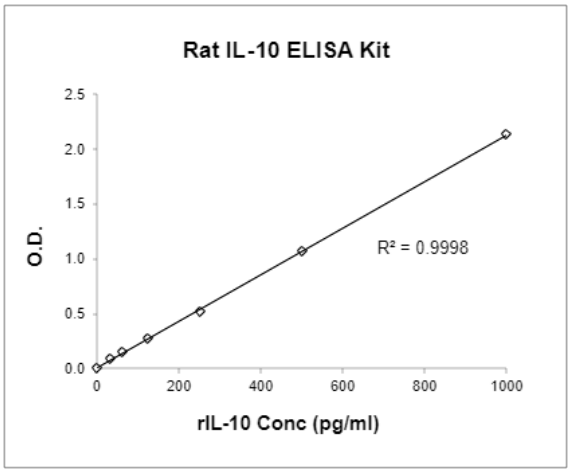
Calculate the average absorbance values for each set of duplicate standards and samples. Ideally duplicates should be within 20% of the mean.

Generate a linear standard curve by plotting the average absorbance of each standard on the vertical axis versus the corresponding rat IL-10 standard concentration on the horizontal axis.

The amount of rat IL-10 in each sample is determined by extrapolating OD values against rat IL-10 standard concentrations using the standard curve**.**

Example rat IL-10 Standard curve

Standard	rIL-10 Conc (pg/ml)	OD (450nm) mean	CV (%)
1	1000.00	2.14	2.3
2	500.00	1.07	1.5
3	250.00	0.53	0.4
4	125.00	0.28	0.8
5	62.50	0.16	1.3
6	31.25	0.09	6.3
zero	0.00	0.02	12.9



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 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-10 defined as the analyte concentration resulting in an absorbance significantly higher than that of the dilution medium (mean plus 2 standard deviations) was determined to be 1.5 pg/ml (mean of 6 independent assays).

12.2. Specificity

The interference of circulating factors of the immune system was evaluated by spiking these proteins at physiologically relevant concentrations into a rat IL-10 positive serum.
There was no cross reactivity detected.

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-10. 2 standard curves were run on each plate. **The calculated overall intra-assay coefficient of variation was < 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-10. 2 standard curves were run on each plate. **The calculated overall inter-assay coefficient of variation was < 10%**.

12.4. Dilution Parallelism

Rat serum samples with different levels of rat IL-10 were analysed at serial 2 fold dilutions with 4 replicates each.
The overall mean recovery was 112%.

13. Assay Summary

Total procedure length : 3h10mn

Wash two times

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Add sample, diluted standard and Biotin-Conjugate

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Incubate 2 hours at room temperature

↓

Wash three times

↓

Add 100µl of Streptavidin-HRP

↓

Incubate 1hour at room temperature

↓

Wash three times

↓

Add 100 µl of ready-to-use TMB
Protect from light. Let the color develop for 10 mn.

↓

Add 100 µl of Stop Reagent

↓

Read Absorbance at 450 nm

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