

Murine IL-5 ELISA Kit

Instructions for use

Catalogue numbers:

1x96 tests: 660.030.096

2x96 tests: 660.030.192

For research use only

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Table of Contents

1.	Intended use	2
2.	Introduction	2
2.1.	Summary	2
2.2.	Principle of the method	2
3.	Reagents provided and reconstitution	3
4.	Materials required but not provided	3
5.	Storage Instructions	4
6.	Specimen collection, processing & storage	4
7.	Safety & precautions for use	5
8.	Assay Preparation	6
8.1.	Assay Design	6
8.2.	Preparation of Wash Buffer	6
8.3.	Preparation of Assay Buffer	6
8.4.	Preparation of Standard	7
8.5.	Preparation of Biotin Conjugate	7
8.6.	Preparation of Streptavidin-HRP	7
9.	Method	8
10.	Data Analysis	9
11.	Assay limitations	9
12.	Performance Characteristics	10
12.1.	Sensitivity	10
12.2.	Specificity	10
12.3.	Precision	10
12.4.	Dilution Parallelism	10
12.5.	Spike Recovery	10
12.6.	Stability	10
12.7.	Expected serum values	10
13.	Bibliography	11
14.	Assay Summary	12

Murine IL-5 ELISA KIT

1. Intended use

The Diaclone Murine IL-5 ELISA kit is a solid phase sandwich ELISA for the *in-vitro* qualitative and quantitative determination of mIL-5 in supernatants, buffered solutions or serum and plasma samples. This assay will recognise both natural and recombinant murine IL-5.

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

2. Introduction

2.1. Summary

Mouse IL-5 has been described as T-cell replacing factor (10,11) and as B-cell growth factor II (9) based on its induction of proliferation and Ig secretion by activated B cells. Several other activities have furthermore been attributed to this cytokine, including induction of growth and differentiation of eosinophils from bone marrow stromal cells (7), induction of mIL-2R expression on both normal splenic and activated B cells (6,4) and enhancements of IgA secretion by LPS-stimulated B-cells (3). m-IL-5 has been shown to be produced exclusively by TH2 subset of mouse TH clones (2). The spectrum of biological activities of mIL-5, on B cells as well as other cell types suggests that mIL-5 plays an important functional role in the activities of TH2 cells. mIL-5 is a heavily glycosylated glycoprotein with native m.w. of 45.000 to 60.000, with multiple subunits of between m.w. 24.000 and 28.000 under reducing conditions (5,1,8).

2.2. Principle of the method

A capture Antibody highly specific for mIL-5 has been coated to the wells of the microtiter strip plate. Binding of mIL-5 samples and known standards to the capture antibodies and subsequent binding of the biotin-conjugated anti-mIL-5 antibody to the analyte is completed during the same incubation period. Any excess unbound analyte and biotin-conjugated 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 stop reagent turning the resultant final product yellow. The intensity of the produced coloured complex is directly proportional to the concentration of mIL-5 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 mIL-5 in any sample tested.

3. Reagents provided and reconstitution

Reagents (Store @ 2-8°C)	Quantity 1x96 well kit Cat no. 660.030.096	Quantity 2x96 well kit Cat no. 660.030.192	Reconstitution
96 well microtiter strip plate	1	2	Ready to use (Pre-coated)
Plastic plate covers	2	4	n/a
mIL-5 Standard: 1000 pg/ml	2	4	Reconstitute as directed on the vial (see Assay preparation, section 8)
Sample Diluent	1 (12ml)	2 (12ml)	Ready to use
Biotin conjugate anti-mIL-5	1 (70µl)	2 (70µl)	Dilute in Assay Buffer 1X (see Assay preparation, section 8)
Streptavidin-HRP	1 (150µl)	2 (150µl)	Dilute in Assay Buffer 1X (see Assay preparation, section 8)
Assay Buffer concentrate	1 (5ml)	2 (5ml)	20x Concentrate dilute in distilled water (see Assay preparation, section 8)
Wash Buffer concentrate	1 (50ml)	2 (50ml)	20x Concentrate dilute in distilled water (see Assay preparation, section 8)
Substrate Solution	1 (15ml)	2 (15ml)	Ready to use
Stop Solution	1 (15ml)	2 (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)
- Microplate 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 30 days.

Assay Buffer 1X: Once prepared, store at 2-8°C for up to 30 days.

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

Diluted Biotin-conjugate Anti-mIL-5: 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.

Plasma: EDTA, citrate and heparin plasma can be assayed. Spin samples at 1000 x g for 30 min to remove particulates. Harvest plasma.

Storage: If not analyzed 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 solution and Substrate Solution. 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 solution and Substrate Solution, 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 TMB solution has been contaminated and must be discarded. Read absorbance's 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 and 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	500	500										
B	250	250										
C	125	125										
D	62.5	62.5										
E	31.3	31.3										
F	15.6	15.6										
G	zero	zero										
H												

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

8.2. Preparation of Wash Buffer

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.

8.3. Preparation of Assay Buffer

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.

Transfer to a clean wash bottle and store at 2° to 8°C.

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 1000 pg/ml of mIL-5. 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 500 to 15.6 pg/ml. A fresh standard curve should be produced for each new assay.

- Add 100µl of Sample Diluent 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 500pg/ml. Mix the well contents by repeated aspirations. 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 500 to 15.6pg/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 directly into the relevant wells.

8.5. Preparation of Biotin Conjugate

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 Buffer (1x) (ml)
1 - 6	0.03	2.97
1 - 12	0.06	5.94

8.6. 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 Buffer (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 8.5) 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.	Preparation	Prepare Standard curve as shown in section 8.4
3.	Addition	Add 100µl of Sample Diluent in duplicate to the blank wells
4.	Addition	Add 50µl of Sample Diluent to all sample wells
5.	Addition	Add 50µl of each sample in duplicate to appropriate number of wells
6.	Addition	Add 50µl of diluted biotin-conjugate to all wells
7.	Incubation	Cover with a plastic plate cover and incubate at room temperature (18 to 25°C) for 2 hour(s) on a microplate shaker set 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 100µl 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 microplate shaker set if available
11.	Wash	Repeat wash step 8.
12.	Addition	Add 100µl of ready-to-use Substrate Solution into all wells
13.	Incubation	Incubate in the dark for 10 minutes* at room temperature. Avoid direct exposure to light by wrapping the plate in aluminium foil.
14.	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 substrate solution 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

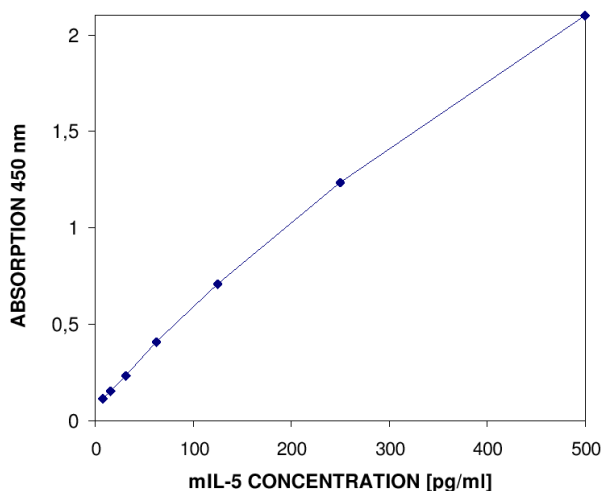
Calculate the average absorbance values for each set of duplicate standards, and samples. Ideally duplicates should be within 20% of the mean.

Generate a linear standard curve by plotting the average absorbance of each standard on the vertical axis versus the corresponding mL-5 standard concentration on the horizontal axis.

The amount of mL-5 in each sample is determined by extrapolating OD values against mL-5 standard concentrations using the standard curve.

Example mL-5 Standard curve

Standard	mL-5 Conc	OD (450nm) mean	CV (%)
1	500	2.099	0.9
2	250	1.233	1.9
3	125	0.705	0.6
4	62.5	0.408	2.6
5	31.3	0.233	2.4
6	15.6	0.153	3.7
zero	0	0.047	-



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 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 mL-5 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 3.3 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 mL-5 positive serum. There was no detectable cross reactivity.

12.3. Precision

Intra-assay

Reproducibility within the assay was evaluated in independent experiments. **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 independent experiments by two technicians. **The overall inter-assay coefficient of variation has been calculated to be <10%.**

12.4. Dilution Parallelism

Murine serum spiked with different levels of mL-5 was assayed at four serial twofold dilutions with 4 replicates each. Experiments showed an overall mean recovery of 93 %.

12.5. Spike Recovery

The spike recovery was evaluated by spiking four levels of mL-5 into pooled normal murine serum. Recoveries were determined in two independent experiments with 4 replicates each. Observed values showed an overall mean recovery of 85%.

12.6. Stability

Storage Stability

Aliquots of spiked serum were stored at -20°C, 2-8°C, room temperature (RT) and at 37°C, and the mL-5 level determined after 24 h. There was no significant loss of mL-5 immunoreactivity during storage at -20°C, 4°C, temperature and 37°C.

Freeze-thaw Stability

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

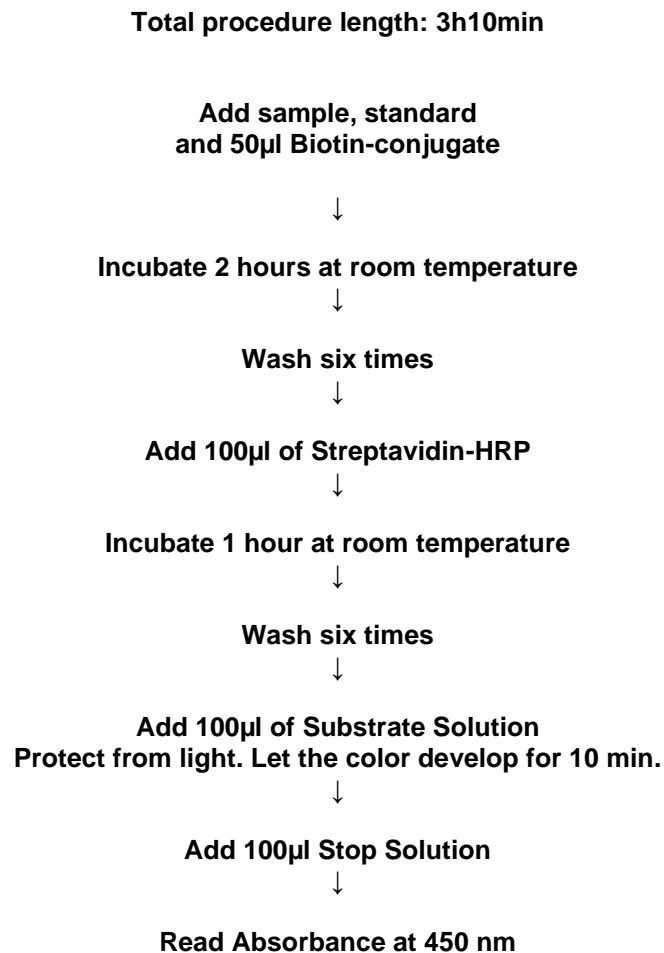
12.7. Expected serum values

There are no detectable mL-5 levels found in healthy mice. Elevated mL-5 levels depend on the type of immunological disorder.

13. Bibliography

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14. Assay Summary



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