Murine Eotaxin ELISA Kit

Instructions for use

Catalogue numbers: 1x96 tests: 660.060.096
2x96 tests: 660.060.192

For research use only

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Version 1 - October 2011

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Murine Eotaxin ELISA KIT

1. Intended use

The mouse Eotaxin ELISA is an enzyme-linked immunosorbent assay for the quantitative detection of mouse Eotaxin. **The mouse Eotaxin ELISA is for research use only. Not for diagnostic or therapeutic procedures.**

2. Introduction

2.1. Summary

Eotaxin, also known as CCL11, is a member of the CC chemokine family of inflammatory and immunoregulatory cytokines.

Constitutive Eotaxin mRNA expression has been detected in multiple tissues, most often it appears to be induced by inflammatory cytokines such as IL-1, TNFα and IFNγ in fibroblasts, endothelial and epithelial cells. Following allergen challenge Eotaxin mRNA has been shown to be rapidly up-regulated in airway epithelium an alveolar macrophages. Eotaxin expression has also been detected in smooth muscle cells, chondrocytes and eosinophils.

Mouse Eotaxin activity is mediated by the mouse CC chemokine receptor CCR3, which, unlike human CCR3, can also be activated by mouse MIP-1α.

Among CC chemokine family members, Eotaxin is functionally and structurally mostly related to the MCP/Eotaxin proteins. Mouse Eotaxin cDNA encodes a 97 amino acid residue precursor protein that is cleaved to generate the 74 aa residue mature protein.

Mouse Eotaxin has been shown to be a potent chemoattractant for eosinophils during inflammation and allergic reactions and to be involved in the growth of myeloid cell progenitors and the differentiation of mast cells during embryonic development. A role in numerous eosinophil-associated gastrointestinals disorders as food allergy, parasitic infections, allergic colitis and inflammatory Bowel disease has been described

2.2. Principle of the method

An anti-mouse Eotaxin coating antibody is adsorbed onto microwells.

Figure 1

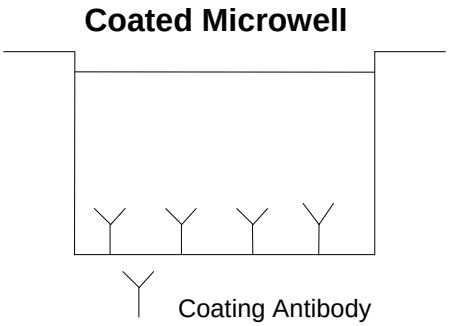


Figure 2

Mouse Eotaxin present in the sample or standard binds to antibodies adsorbed to the microwells. A biotin-conjugated anti-mouse Eotaxin antibody is added and binds to mouse Eotaxin captured by the first antibody.

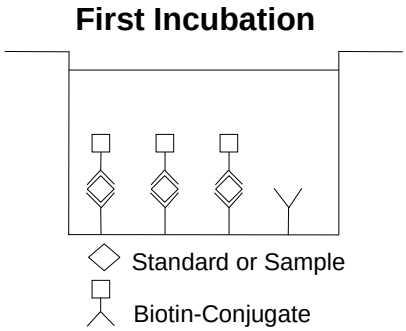


Figure 3

Following incubation unbound biotin-conjugated anti-mouse Eotaxin antibody is removed during a wash step. Streptavidin-HRP is added and binds to the biotin-conjugated anti-mouse Eotaxin antibody.

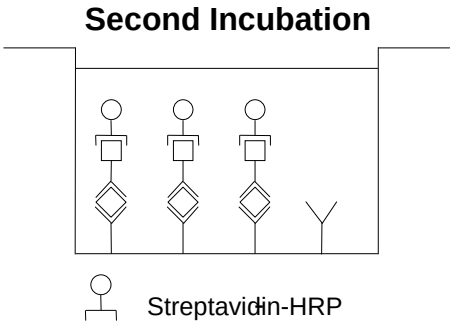
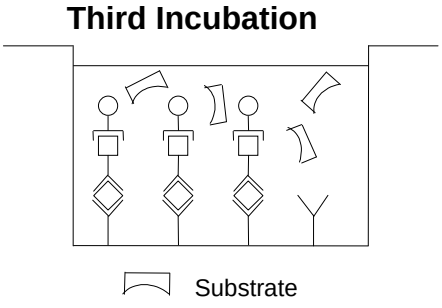


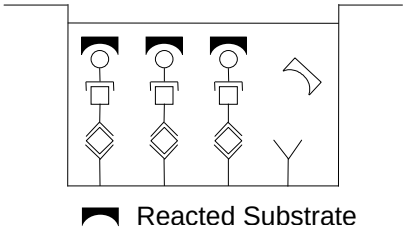
Figure 4

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 mouse Eotaxin present in the sample or standard. The reaction is terminated by addition of acid and absorbance is measured at 450 nm. A standard curve is prepared from 7 mouse Eotaxin standard dilutions and mouse Eotaxin sample concentration determined.

Figure 5



3. Reagents provided and reconstitution

(Note: Quantity shown is for the 1x96 format, for the 2x96 format all reagents will be supplied in duplicate)

1 aluminium pouch with a **Microwell Plate coated** with polyclonal antibody to mouse Eotaxin
1 vial (100 µl) **Biotin-Conjugate** anti-mouse Eotaxin polyclonal antibody
1 vial (150 µl) **Streptavidin-HRP**
2 vials mouse Eotaxin **Standard** lyophilized, 4 ng/ml upon reconstitution
1 vial (12 ml) **Sample Diluent**
1 vial (5 ml) **Assay Buffer Concentrate** 20x (PBS with 1% Tween 20 and 10% BSA)
1 bottle (50 ml) **Wash Buffer Concentrate** 20x (PBS with 1% Tween 20)
1 vial (15 ml) **Substrate Solution** (tetramethyl-benzidine)
1 vial (15 ml) **Stop Solution** (1M Phosphoric acid)
1 vial (0.4 ml) **Blue-Dye**
1 vial (0.4 ml) **Green-Dye**
1 vial (0.4 ml) **Red-Dye**
4 **Adhesive Films**

4. Materials required but not provided

- Microtitre plate reader fitted with appropriate filters (450nm required with optional 620nm 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° to 8°C). Expiry of the kit and reagents is stated on labels.
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, this reagent is not contaminated by the first handling.

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 mouse Eotaxin. 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.

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 H_2SO_4 and TMB. 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 or frozen 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 H_2SO_4 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 TMB solution is light sensitive. Avoid prolonged exposure to light. Also, avoid contact of the TMB solution with metal to prevent colour development. Warning TMB 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 TMB solution within 15 min of the washing of the microtitre 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 aluminium 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^{o}C storage.

Example plate layout (example shown for a 7 point standard curve)

	Standards		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	Zero	Zero										

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. Please note that Wash Buffer (1x) is stable for 30 days.

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

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. Please note that the Assay Buffer (1x) is stable for 30 days.

Assay Buffer (1x) may also be prepared as needed according to the following table:

Number of Strips	Assay Buffer Concentrate (20x) (ml)	Distilled Water (ml)
1 - 6	2.5	47.5
1 - 12	5.0	95.0

8.4. Preparation of Standard

Reconstitute **mouse Eotaxin standard** by addition of distilled water.

Reconstitution volume is stated on the label of the standard vial. Allow the reconstituted standard to sit for 10-30 minutes. Swirl or mix gently to insure complete and homogeneous solubilization (concentration of reconstituted standard = 4000 pg/ml).

The standard has to be used immediately after reconstitution and cannot be stored.

After reconstitution Add 100 µl of Sample Diluent in duplicate to all **standard wells**. Pipette 100 µl of prepared **standard** in duplicate into well A1 and A2. Mix the contents of wells A1 and A2 by repeated aspiration and ejection (concentration of standard 1, S1 = 2000 pg/ml), and transfer 100 µl to wells B1 and B2, respectively. Take care not to scratch the inner surface of the microwells. Continue this procedure 5 times, creating two rows of mouse Eotaxin standard dilutions ranging from 2000 to 31.3 pg/ml. Discard 100 µl of the contents from the last microwells (G1, G2) used.

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

8.7. Addition of Colour-giving Reagents: Blue-Dye, Green-Dye, Red-Dye

In order to help our customers to avoid any mistakes in pipetting this ELISA, we offer a 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:

Diluent: Before standard and 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 Sample Diluent	20 µl ***Blue-Dye***
12 ml Sample Diluent	48 µl ***Blue-Dye***
50 ml Sample Diluent	200 µl ***Blue-Dye***

Biotin-Conjugate: Before dilution of the concentrated Biotin-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 (1x)	30 µl ***Green-Dye***
6 ml Assay Buffer (1x)	60 µl ***Green-Dye***

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 (1x)	24 µl ***Red-Dye***
12 ml Assay Buffer (1x)	48 µl ***Red-Dye***

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	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
2.	Addition	**Prepare Standard curve** as shown in section 8.4
3.	Addition	Add 100µl of each **Standard, Control (where applicable) and zero (appropriate sample diluent)** in duplicate to appropriate number of wells
4.	Addition	Add 50µl of **Sample diluent** and 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 three times
8.	Addition	Add 100µl of **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 7.
11.	Addition	Add 100µl of ready-to-use **TMB 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 Reagent** into all wells
Read the absorbance value of each well (immediately after step 13.) 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*

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. Duplicates should be within 20 per cent of the mean value.

Create a standard curve by plotting the mean absorbance for each standard concentration on the ordinate against the mouse Eotaxin concentration on the abscissa. Draw a best fit curve through the points of the graph (a 5-parameter curve fit is recommended).

To determine the concentration of circulating mouse Eotaxin 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 mouse Eotaxin concentration.

If instructions in this protocol have been followed samples have been diluted 1:2 (50 µl sample + 50 µl Sample Diluent), the concentration read from the standard curve must be multiplied by the dilution factor (x 2).

Calculation of samples with a concentration exceeding standard 1 may result in incorrect, low mouse Eotaxin levels. Such samples require further external predilution according to expected mouse Eotaxin values with Sample Diluent in order to precisely quantitate the actual mouse Eotaxin level.

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

Figure 6
Representative standard curve for mouse Eotaxin ELISA. Mouse Eotaxin was diluted in serial 2-fold steps in Sample Diluent. Do not use this standard curve to derive test results. A standard curve must be run for each group of microwell strips assayed.

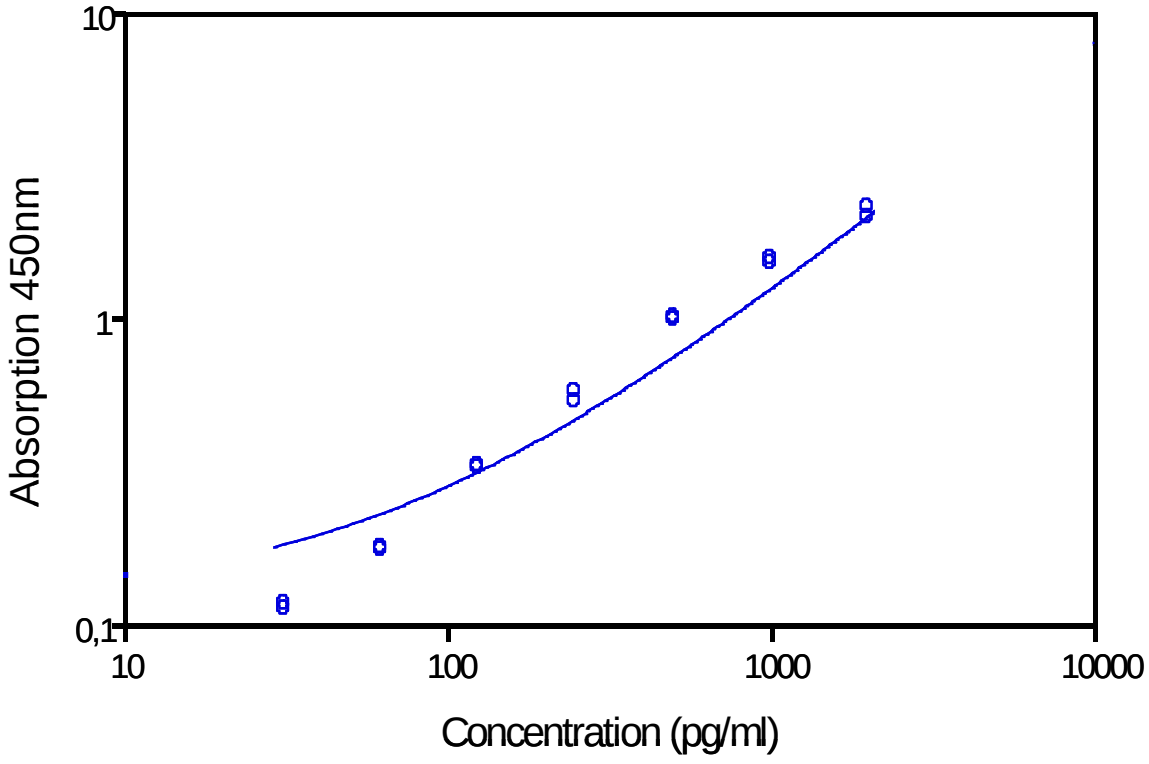


Table 1
Typical data using the mouse Eotaxin ELISA
Measuring wavelength: 450 nm
Reference wavelength: 620 nm

Standard	Mouse Eotaxin Concentration (pg/ml)	O.D. at 450 nm	Mean O.D. at 450 nm	C.V. (%)
1	2000.0	2.306 2.149	2.228	5.0
2	1000.0	1.509 1.577	1.543	3.1
3	500.0	1.001 0.981	0.991	1.4
4	250.0	0.534 0.573	0.554	5.0
5	125.0	0.323 0.329	0.326	1.3
6	62.5	0.176 0.178	0.177	1.0
7	31.3	0.112 0.117	0.115	3.2
Blank	0.0	0.041 0.036	0.039	9.2

The OD values of the standard curve may vary according to the conditions of assay performance (e.g. operator, pipetting technique, washing technique or temperature effects). Furthermore shelf life of the kit may affect enzymatic activity and thus colour intensity. Values measured are still valid.

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 Washing Buffer, fill with Washing 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 mouse Eotaxin 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 3.6 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 mouse Eotaxin positive serum.
There was no crossreactivity 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 8 serum samples containing different concentrations of mouse Eotaxin. 2 standard curves were run on each plate. Data below show the mean mouse Eotaxin concentration and the coefficient of variation for each sample (see Table). **The calculated overall intra-assay coefficient of variation was 5.6%.**

Table 2
The mean mouse Eotaxin concentration and the coefficient of variation for each sample

Sample	Experiment	Mean Mouse Eotaxin Concentration (pg/ml)	Coefficient of Variation (%)
1	1	749.6	9.0
	2	761.4	6.0
	3	812.6	7.0
2	1	326.1	3.0
	2	293.3	4.0
	3	335.1	5.0
3	1	160.3	5.0
	2	156.1	4.0
	3	156.6	4.0
4	1	136.7	4.0
	2	118.3	6.0
	3	130.3	7.0
5	1	950.3	10.0
	2	685.9	3.0
	3	839.9	7.0
6	1	312.9	10.0
	2	260.0	4.0
	3	330.1	5.0
7	1	160.1	6.0
	2	137.1	6.0
	3	170.6	4.0
8	1	155.7	5.0
	2	112.4	7.0
	3	138.1	6.0

Inter-assay

Assay to assay reproducibility within one laboratory was evaluated in 3 independent experiments. Each assay was carried out with 6 replicates of 8 serum samples containing different concentrations of mouse Eotaxin. 2 standard curves were run on each plate. Data below show the mean mouse Eotaxin concentration and the coefficient of variation calculated on 18 determinations of each sample (see Table).
The calculated overall inter-assay coefficient of variation was 9.4%.

Table 3
The mean mouse Eotaxin concentration and the coefficient of variation of each sample

Sample	Mean Mouse Eotaxin Concentration (pg/ml)	Coefficient of Variation (%)
1	774.5	4.3
2	318.2	6.9
3	157.7	1.4
4	128.5	7.3
5	825.4	16.1
6	301.0	12.1
7	156.0	11.0
8	135.4	16.1

12.4. Dilution Parallelism

Serum samples with different levels of mouse Eotaxin were analysed at serial 2 fold dilutions with 4 replicates each.
The recovery ranged from 103% to 121% with an overall recovery of 114%

12.5. Spike Recovery

The spike recovery was evaluated by spiking 4 levels of mouse Eotaxin into serum. Recoveries were determined in 3 independent experiments with 6 replicates each. The unspiked serum was used as blank in these experiments.
The overall mean recovery was 66%.

12.6. Stability

Freeze-Thaw Stability

Aliquots of serum samples were stored at -20°C and thawed 5 times, and the mouse Eotaxin levels determined. There was no significant loss of mouse Eotaxin immunoreactivity detected by freezing and thawing.

Storage Stability

Aliquots of serum samples were stored at -20°C, 2-8°C, room temperature (RT) and at 37°C, and the mouse Eotaxin level determined after 24 h. There was no significant loss of mouse Eotaxin immunoreactivity detected during storage under above conditions.

13. Assay Summary

Total procedure length : 3h10mn

Wash two times

↓

Add sample, diluted standard and Biotin-Conjugate

↓

Incubate 2 hours at room temperature

↓

Wash four times

↓

Add 100µl of Streptavidin-HRP

↓

Incubate 1hour at room temperature

↓

Wash four times

↓

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

↓

Add 100 µl Stop Reagent

↓

Read Absorbance at 450 nm

Supplier:

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