ELISA Kit

Catalog # KAC2231 (96 tests)

Human Eotaxin

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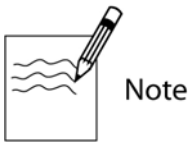
Contents and Storage

Storage

Store at 2 to 8°C.

Contents

Reagents Provided	*96 Test Kit*
Hu Eotaxin Standard, lyophilized, recombinant Hu Eotaxin. Refer to vial label for quantity and reconstitution volume.	2 vials
Standard Diluent Buffer. Contains 7.7 mM sodium azide; 25 mL per bottle.	1 bottle
Hu Eotaxin Antibody-Coated Wells, 96 wells per plate.	1 plate
Hu Eotaxin Biotin Conjugate (Biotin-labeled anti-Eotaxin). Contains 7.7 mM sodium azide; 11 mL per bottle.	1 bottle
Streptavidin-Peroxidase (HRP), (100x concentrate). Contains 0.04% Proclin® 300, 3.3 mM thymol; 0.125 mL per vial.	1 vial
Streptavidin-Peroxidase (HRP) Diluent. Contains 0.04% Proclin® 300 and 3.3 mM thymol; 25 mL per bottle.	1 bottle
Wash Buffer Concentrate (25x); 100 mL per bottle.	1 bottle
Incubation Buffer. Contains 7.7 mM sodium azide; 11 mL per bottle.	1 bottle
Stabilized Chromogen, Tetramethylbenzidine (TMB); 25 mL per bottle.	1 bottle
Stop Solution; 25 mL per bottle.	1 bottle
Plate Covers, adhesive strips.	3



Disposal Note: This kit contains materials with small quantities of sodium azide and Proclin® 300. Sodium azide reacts with lead and copper plumbing to form explosive metal azides. Upon disposal, flush drains with a large volume of water to prevent azide accumulation. Proclin® 300 is toxic. Avoid ingestion and contact with eyes, skin and mucous membranes. In case of contact, rinse affected area with plenty of water. Observe all federal, state and local regulations for disposal.

Safety

All blood components and biological materials should be handled as potentially hazardous. Follow universal precautions as established by the Centers for Disease Control and Prevention and by the Occupational Safety and Health Administration when handling and disposing of infectious agents.

Introduction

Intended Use

The Invitrogen Human Eotaxin (Hu Eotaxin) ELISA is to be used for the *in vitro* quantitative determination of Hu Eotaxin in human serum, EDTA plasma, buffered solution, or cell culture medium. Heparinized plasma is not recommended in this assay. The assay will recognize both natural and recombinant Hu Eotaxin.

Principle of the Method

The Invitrogen Hu Eotaxin kit is a solid phase sandwich Enzyme Linked-Immuno-Sorbent Assay (ELISA). A monoclonal antibody specific for Hu Eotaxin has been coated onto the wells of the microtiter strips provided. Samples, including standards of known Hu Eotaxin content, control specimens, and unknowns, are pipetted into these wells, followed by the addition of a biotinylated monoclonal second antibody.

During the first incubation, the Hu Eotaxin antigen binds simultaneously to the immobilized (capture) antibody on one site, and to the solution phase biotinylated antibody on a second site.

After removal of excess second antibody, Streptavidin-Peroxidase (enzyme) is added. This binds to the biotinylated antibody to complete the four-member sandwich. After a second incubation and washing to remove all the unbound enzyme, a substrate solution is added, which is acted upon by the bound enzyme to produce color. The intensity of this colored product is directly proportional to the concentration of Hu Eotaxin present in the original specimen.

Background Information

Human Eotaxin is an 8.3 kDa, 73 amino acid, non-glycosylated polypeptide (1,2). Eotaxin was initially identified by its biological activity in the broncho-alveolar lavage fluid of guinea pigs that were used in an allergic inflammation model (3,4). Genetic analysis indicates that Eotaxin is closely related to MCPs (Monocyte Chemotactic Proteins) and thus is considered to be a member of the ß (or CC) chemokine subfamily. The CC chemokines, in which the first two cysteine residues are adjacent, are typically chemoattractants for monocytes, eosinophils, basophils, and lymphocytes with variable selectivity. Eotaxin exhibits an inter-species homology of 60% between human, mouse and guinea pig (5-8). In humans, Eotaxin shows 50-70% identity at the amino acid and nucleotide level with MCP-1, MCP-2 and MCP-4, ~30% with RANTES and MIP-1α, and ~30% with HCC-1. (1,9,10) A functionally similar factor, Eotaxin-2, shares only ~35% amino acid identity with Eotaxin (11,12).

Chemokines mediate their action by binding to specific cell surface receptors that are members of a large family of G-protein-coupled, seven transmembrane domain receptors (13). Receptors that bind CC chemokines are designated CCRs, with 9 CCRs identified to date. Chemokine receptors are known to exhibit overlapping ligand specificity (i.e., a given leukocyte often expresses multiple chemokine receptors and more than one chemokine typically binds to the same receptor). Eotaxin (and Eotaxin-2) are exceptional in this regard, since they bind only to chemokine receptor, CCR3. In contrast, MCP-2, -3, -4 and RANTES are ligands for multiple receptors including CCR3 (14-16). Cells known to express CCR3 include eosinophils, basophils, and Th2-type T cells. Human eosinophils express CCR3 at ~350,000 sites per cell (17,18). The restrictive receptor-specificity of Eotaxin to CCR3 and the predominant expression of CCR3 on human eosinophils explain why Eotaxin acts as an eosinophil-selective chemokine.

Eotaxin and related MCPs are likely to play a critical role in the regulation of the inflammatory characteristics of allergic disease (19). The *in vitro* and *in vivo* involvement of chemokines in the process of eosinophil accumulation was demonstrated in several studies (2,7,20). Eotaxin plays an important role in stimulating the local recruitment of eosinophils from blood microvessels into the tissue at sites of allergic inflammation (2,6,20,21). Eotaxin is believed to regulate leukocyte chemoattraction and cellular activation. While Eotaxin may be induced in many situations where inflammatory cytokines like TNF, IL-1, and IFN-γ are present, only in those situations where IL-5 is co-produced will tissue eosinophilia result (7,22,23). Since circulating levels of eosinophils are normally low, Eotaxin may contribute to the mobilization of hematopoietic progenitors and to their differentiation into mature eosinophils, essential for the induction of allergic reactions (24).

Methods

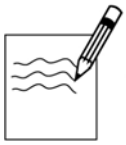
Materials Needed But Not Provided

- Microtiter plate reader (at or near 450 nm) with software
- Calibrated adjustable precision pipettes
- Distilled or deionized water
- Plate washer: automated or manual (squirt bottle, manifold dispenser, etc.)
- Glass or plastic tubes for diluting solutions
- Absorbent paper towels
- Calibrated beakers and graduated cylinders

Procedural Notes

1. When not in use, kit components should be refrigerated. All reagents should be warmed to room temperature before use.
2. **Microtiter plates should be allowed to come to room temperature before opening the foil bags.** Once the desired number of strips has been removed, immediately reseal the bag and store at 2 to 8°C to maintain plate integrity.
3. Samples should be collected in pyrogen/endotoxin-free tubes.
4. Samples should be frozen if not analyzed shortly after collection. Avoid multiple freeze-thaw cycles of frozen samples. Thaw completely and mix well prior to analysis.
5. When possible, avoid use of badly hemolyzed or lipemic sera. If large amounts of particulate matter are present, centrifuge or filter prior to analysis.
6. It is recommended that all standards, controls and samples be run in duplicate.
7. Samples above the highest standard should be diluted with *Standard Diluent Buffer*.
8. When pipetting reagents, maintain a consistent order of addition from well-to-well. This ensures equal incubation times for all wells.
9. **Do not mix or interchange different reagent lots from various kit lots**.
10. Do not use reagents after the kit expiration date.
11. Absorbances should be read immediately, but can be read up to 2 hours of assay completion. For best results, keep plate covered in the dark.
12. In-house controls should be run with every assay. If control values fall outside pre-established ranges, the accuracy of the assay is suspect.
13. All residual wash 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.
14. Because Stabilized *Chromogen* is light sensitive, avoid prolonged exposure to light. Avoid contact between chromogen and metal, or color may develop.

Directions for Washing



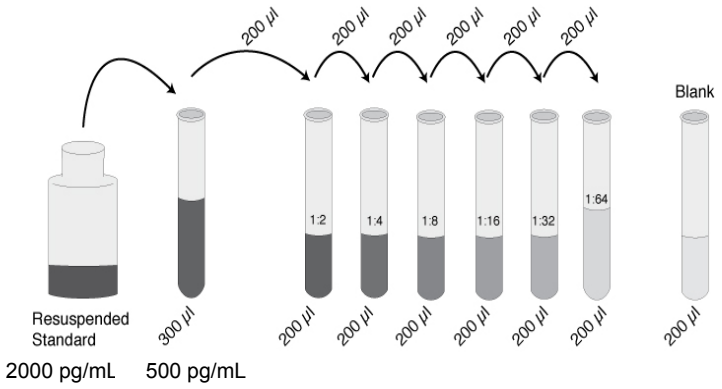
- **Incomplete washing will adversely affect the test outcome**. All washing must be performed with Wash Buffer provided.
- Washing can be performed manually as follows: completely aspirate the liquid from all wells by gently lowering an aspiration tip into the bottom of each well. Take care not to scratch the inside of the well. After aspiration, fill the wells with at least 0.4 mL of diluted wash solution. Let soak for 15 to 30 seconds, then aspirate the liquid. Repeat as directed under Assay Procedure. After the washing procedure, the plate is inverted and tapped dry on absorbent tissue.
- Alternatively, the wash solution may be put into a squirt bottle. If a squirt bottle is used, flood the plate with wash buffer, completely filling all wells. After the washing procedure, the plate is inverted and tapped dry on absorbent tissue.
- If using an automated washer, follow the operating washing instructions carefully.

Preparation of Reagents

Dilution of Standard

Important

1. Reconstitute standard to 2000 pg/mL with *Standard Diluent Buffer*. Refer to standard vial label for instructions. Swirl or mix gently and allow to sit for 10 minutes to ensure complete reconstitution. Use standard within 1 hour of reconstitution.
2. Add 0.100 mL of the reconstituted standard to a tube containing 0.300 mL *Standard Diluent Buffer*. Label as 500 pg/mL Hu Eotaxin. Mix by pipetting up and down.
3. Add 0.200 mL of *Standard Diluent Buffer* to each of 6 tubes labeled 250, 125, 62.5, 31.2, 15.6, and 7.8 pg/mL Hu Eotaxin.
4. Make serial dilutions of the standard as described in the following dilution diagram. Mix thoroughly between steps.
5. Discard all remaining reconstituted and diluted standards after completing assay. Return the *Standard Diluent Buffer* to the refrigerator.



Preparing SAV-HRP

Note

1. Dilute 10 µL of this 100x concentrated solution with 1 mL of *Streptavidin-HRP Diluent* for each 8-well strip used in the assay. Label as Streptavidin-HRP Working Solution.
2. Return the unused *Streptavidin-HRP* concentrate to the refrigerator.

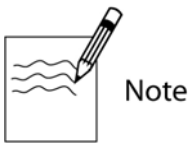
Note: Prepare within 15 minutes of usage, as activity decreases.

# of 8-Well Strips	Volume of Streptavidin-HRP Concentrate	Volume of Diluent
2	20 µL solution	2 mL
4	40 µL solution	4 mL
6	60 µL solution	6 mL
8	80 µL solution	8 mL
10	100 µL solution	10 mL
12	120 µL solution	12 mL

Dilution of Wash Buffer

1. Allow the 25x concentrate to reach room temperature and mix to ensure that any precipitated salts have redissolved. Dilute 1 volume of the 25x wash buffer concentrate with 24 volumes of deionized water (e.g., 50 mL may be diluted up to 1.25 liters, 100 mL may be diluted up to 2.5 liters). Label as Working Wash Buffer.
2. Store both the concentrate and the Working Wash Buffer in the refrigerator. The diluted buffer should be used within 14 days.

Assay Procedure



Be sure to read the *Procedural Notes* section before carrying out the assay.

Allow all reagents to reach room temperature before use. Gently mix all liquid reagents prior to use.

Note: A standard curve must be run with each assay.

1. Determine the number of 8-well strips needed for the assay. Insert these in the frame(s) for current use. (Re-bag extra strips and frame. Store these in the refrigerator for future use.)
2. Add 50 µL of the *Incubation Buffer* to the wells reserved for standards, and serum/plasma samples.
3. Add 50 µL of the *Standard Diluent Buffer* to the wells reserved for cell culture samples.
4. Add 50 L of the *Standard Diluent Buffer* to zero standard wells. Well(s) reserved for chromogen blank should be left empty.
5. Add 50 µL of standards, samples or controls to the appropriate microtiter wells. See **Dilution of Standards**.
6. Pipette 100 µL of biotinylated anti-Eotaxin (*Biotin Conjugate*) solution into each well except the chromogen blank(s). Tap gently on the side of the plate to mix.
7. Cover plate with *plate cover* and incubate for **2 hours at room temperature**.
8. Thoroughly aspirate or decant solution from wells and discard the liquid. Wash wells 4 times. See **Directions for Washing.**
9. Add 100 µL Streptavidin-HRP Working Solution to each well except the chromogen blank(s). Prepare the working dilution as described in **Preparing SAV-HRP**.
10. Cover plate with the *plate cover* and incubate for **30 minutes at room temperature**.
11. Thoroughly aspirate or decant solution from wells and discard the liquid. Wash wells 4 times. See **Directions for Washing.**
12. Add 100 µL of *Stabilized Chromogen* to each well. (Color of chromogen should be clear prior to adding it to the well). The liquid in the wells will begin to turn blue.
13. Incubate for **30 minutes at room temperature and in the dark**. ***Please Note*: Do not cover the plate with aluminum foil or metalized mylar**. The incubation time for chromogen substrate is often determined by the microtiter plate reader used. Many plate readers have the capacity to record a maximum optical density (O.D.) of 2.0. The O.D. values should be monitored and the substrate reaction stopped before the O.D. of the positive wells exceed the limits of the instrument. If using a reader that records only to 2.0 O.D., stopping the assay after 20 to 25 minutes is suggested.
14. Add 100 µL of *Stop Solution* to each well. Tap side of plate gently to mix. The solution in the wells should change from blue to yellow.
15. Read the absorbance of each well at 450 nm having blanked the plate reader against a chromogen blank composed of 100 µL each of *Stabilized Chromogen* and *Stop Solution*. Read the plate within 2 hours after adding the *Stop Solution*.
16. Use a curve fitting software to generate the standard curve. A four parameter algorithm provides the best standard curve fit.
17. Read the concentrations for unknown samples and controls from the standard curve. (Samples producing signals greater than that of the highest standard should be diluted in *Standard Diluent Buffer* and reanalyzed, multiplying the concentration found by the appropriate dilution factor.)

Typical Data (Example)

The following data were obtained for the various standards over the range of 0 to 500 pg/mL Hu Eotaxin.

Standard Hu Eotaxin (pg/mL)	Optical Density (450 nm)
0	0.045 0.042
7.8	0.095 0.092
15.6	0.146 0.148
31.2	0.269 0.248
62.5	0.450 0.459
125	0.884 0.888
250	1.574 1.598
500	3.151 3.142

Performance Characteristics

Sensitivity

The minimum detectable dose of Hu Eotaxin is <2.2 pg/mL. This was determined by adding two standard deviations to the mean O.D. obtained when the zero standard was assayed 32 times.

Precision

1. Intra-Assay Precision

Serum-based and buffer-based samples of known Hu Eotaxin concentration were assayed in replicates of 16 to determine precision within an assay.

	Sample 1	Sample 2	Sample 3
Mean (pg/mL)	92.0	219.4	375.1
SD	3.8	10.9	13.9
%CV	4.1	4.9	3.7

SD = Standard Deviation
CV = Coefficient of Variation

2. Inter-Assay Precision

Samples were assayed 8 times in 5 different assays to determine precision between assays.

	Sample 1	Sample 2	Sample 3
Mean (pg/mL)	85.4	203.4	345.3
SD	3.5	10.0	20.1
%CV	4.0	4.9	5.8

SD = Standard Deviation
CV = Coefficient of Variation

Linearity of Dilution

Human serum containing 222 pg/mL of measured Hu Eotaxin was serially diluted in Standard Diluent Buffer over the range of the assay. Linear regression analysis of samples versus the expected concentration yielded an average correlation coefficient of 0.999.

Recovery

The recovery of Hu Eotaxin added to normal human serum and normal human plasma averaged 100% and 88%, respectively. The recovery of Hu Eotaxin added to tissue culture medium containing 1% and 10% fetal bovine serum averaged 85% and 90%, respectively.

Specificity

Buffered solutions of a panel of substances at 150 ng/mL were assayed with the Hu Eotaxin kit. The following substances were tested and found to have no cross-reactivity: human IL-1α, IL-1ß, IL-2, IL-3, IL-4, IL-5, IL-6, IL-8, IL-9, IL-10, IL-12, IL-16, G-CSF, M-CSF, GM-CSF, GRO, SCF, IP-10, IFN-α, IFN-γ, LIF, MIP-1α, MIP-1ß, MCP-1, MCP-3, MCP-4, OSM, RANTES, TNF-α, EGF.

Expected Values

Each laboratory must establish its own normal values. For guidance, the mean of 16 normal sera was 133 pg/mL (SD=40.9), ranging between 71.2 and 247.8 pg/mL. The mean of EDTA plasma were 74.1 pg/mL (SD=23.6), ranging between 42.1 and 141.4 pg/mL. The mean of 16 normal citrate plasma was 138 pg/mL (SD=40.8), ranging between 85.4 and 209.2 pg/mL. No Eotaxin was detectable from human peripheral blood mononuclear cells cultured for up to 72 hours in RPMI supplemented with 5% FCS, after a variety of stimulation conditions including PMA/ionophore (0.1 μg/mL each) or PHA (5 μg/mL), and LPS (25 μg/mL).

Limitations of the Procedure

Do not extrapolate the standard curve beyond the top standard point; the dose-response is non-linear in this region and accuracy is difficult to obtain. Dilute all samples above the top standard point with *Standard Diluent Buffer*; reanalyze these and multiply results by the appropriate dilution factor.

The influence of various drugs, aberrant sera (hemolyzed, hyperlipidemic, jaundiced, etc.) and the use of biological fluids in place of serum samples have not been thoroughly investigated. The rate of degradation of native Hu Eotaxin in various matrices has not been investigated. The immunoassay literature contains frequent references to aberrant signals seen with some sera, attributed to heterophilic antibodies. Though such samples have not been seen to date, the possibility of this occurrence cannot be excluded.

Appendix

Troubleshooting Guide

Elevated background	*Cause:* Insufficient washing and/or draining of wells after washing. Solution containing either biotin or SAV-HRP can elevate the background if residual is left in the well. *Solution:* Wash according to the protocol. Verify the function of automated plate washer. At the end of each washing step, invert plate on absorbent tissue on countertop and allow to completely drain and tap forcefully if necessary to remove residual. *Cause:* Contamination of substrate solution with metal ions or oxidizing reagents. *Solution:* Use distilled/deionized water for dilution of wash buffer and use plastic equipment. DO NOT COVER plate with foil. *Cause:* Contamination of pipette, dispensing reservoir or substrate solution with SAV-HRP conjugate. *Solution:* Do not use chromogen that appears blue prior to dispensing onto the plate. Obtain new vial of chromogen. *Cause:* Incubation time is too long or incubation temperature is too high. *Solution:* Reduce incubation time and/or temperature.
Elevated sample/ standard ODs	*Cause:* Incorrect dilution of standard stock solution; intermediary dilutions not followed correctly. *Solution*: Follow the protocol instructions regarding the dilution of the standard. *Cause:* Incorrect dilution of the SAV-HRP conjugate. *Solution:* Warm solution of SAV-HRP concentrate to room temperature, draw up slowly and wipe tip with kim-wipe to remove excess. Dilute ONLY in SAV diluent provided. *Cause*: Incubation times extended. *Solution:* Follow incubation times outlined in protocol. *Cause:* Incubations carried out at 37°C when RT is dictated. *Solution:* Perform incubations at RT (= 25 ± 2°C) when instructed in the protocol.
Poor standard curve	*Cause:* Improper preparation of standard stock solution. *Solution:* Dilute lyophilized standard as directed by the vial label only with the standard diluent buffer or in a diluent that most closely matches the matrix of your sample. *Cause:* Reagents (lyophilized standard, standard diluent buffer, etc.) from different kits, either different cytokine or different lot number, were substituted. *Solution:* NEVER substitute any components from another kit. *Cause*: Errors in pipetting the standard or subsequent steps. *Solution*: Always dispense into wells quickly and in the same order. Do not touch the pipette tip on the individual microwells when dispensing. Use calibrated pipettes and the appropriate tips for that device.

Weak/no color develops	*Cause:* Reagents not at RT (25 ± 2°C) at start of assay. *Solution:* Allow ALL reagents to warm to RT prior to commencing assay. *Cause:* Incorrect storage of components, e.g., not stored at 2 to 8°C. *Solution:* Store all components exactly as directed in protocol and on labels. *Cause:* Working SAV-HRP solution made up longer than 15 minutes before use in assay. *Solution:* Use the diluted SAV-HRP within 15 minutes of dilution. *Cause:* TMB solution lost activity. *Solution 1:* The TMB solution should be clear before it is dispensed into the wells of the microtiter plate. An intense aqua blue color indicates that the product is contaminated. Please contact Technical Support if this problem is noted. To avoid contamination, we recommend that the quantity required for an assay be dispensed into a disposable trough for pipetting. Any TMB solution left in the trough should be discarded. *Solution 2:* Avoid contact of the TMB solution with items containing metal ions. *Cause:* Attempt to measure analyte in a matrix for which the ELISA assay has not been optimized. *Solution:* Please contact Technical Support for advice when using nonvalidated sample types. *Cause:* Wells have been scratched with pipette tip or washing tips. *Solution:* Use caution when dispensing and aspirating into and out of microwells.
Poor Precision	*Cause:* Errors in pipetting the standards, samples or subsequent steps. *Solution:* Always dispense into wells quickly and in the same order. Do not touch the pipette tip on the individual microwells when dispensing. Use calibrated pipettes and the appropriate tips for that device. Check for any leaks in the pipette tip. *Cause:* Repetitive use of tips for several samples or different reagents. *Solution:* Use fresh tips for each sample or reagent transfer. *Cause:* Wells have been scratched with pipette tip or washing tips. *Solution:* Use caution when dispensing and aspirating into and out of microwells.

Technical Support

Contact Us

For more troubleshooting tips, information, or assistance, please call, email, or go online to www.invitrogen.com/ELISA.

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Human Eotaxin Assay Summary