Quantikine® ELISA

Human CCL26/Eotaxin-3 Immunoassay

Catalog Number DCC260

For the quantitative determination of human Eotaxin-3 concentrations in cell culture supernates, serum, and plasma.

Note: The standard reconstitution method has changed. Please read this package insert in its entirety before using this product.

This package insert must be read in its entirety before using this product.
For research use only. Not for use in diagnostic procedures.

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614 McKinley Place NE, Minneapolis, MN 55413, USA
TEL: (800) 343-7475 (612) 379-2956 FAX: (612) 656-4400
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INTRODUCTION

Eotaxin-3 (also known as CCL26 or SCYA26) is a CC chemokine with potent chemotactic activity for eosinophils (1-4). It was originally cloned from the GenBank human expressed sequence tag (EST) database (1). Eotaxin-3, along with Eotaxin-1 and Eotaxin-2, selectively activates the CC chemokine receptor 3 (CCR3). The Eotaxin-3-CCR3 interaction may play an important role in allergic diseases such as atopic dermatitis and bronchial asthma (5).

The full-length cDNA for Eotaxin-3 encodes a protein of 94 amino acids (aa) with a putative signal peptide of either 23 or 26 aa residues (1, 2). Both the 71 and 68 aa residue variants of recombinant Eotaxin-3 demonstrate equal potency in inducing chemotaxis of a human CCR3-transfected cell line (2). The mature Eotaxin-3 protein demonstrates 44% aa identity with MIP-1β and 40% aa identity with MIP-1α, RANTES and MCP-4 (1). Unlike most other CC chemokines, Eotaxin-3 maps to human chromosome 7q11.2, within 40 kilobases of the Eotaxin-2 loci (1, 2). Eotaxin-3 and Eotaxin-2 are unique in that they are the only chemokines identified to date that map to chromosome 7 (2, 6).

Like Eotaxin-1 and Eotaxin-2, Eotaxin-3 is a ligand for CCR3. The potency of Eotaxin-3 as a CCR3 ligand, however, is ten-fold less than that of Eotaxin-1 (2). CCR3 is a seven-transmembrane-spanning G-protein-linked receptor expressed on eosinophils, basophils, subpopulations of Th2 lymphocytes, and keratinocytes (5). Signal transduction via CCR3 is characterized by actin polymerization, a transient rise in cytosolic calcium concentration, and release of reactive oxygen species. Other chemokines capable of signaling through CCR3 include Eotaxin-1, Eotaxin-2, MCP-2, MCP-3, MCP-4, MIP-1δ, and RANTES. Studies indicate that Eotaxin-3 is capable of cross-desensitizing cells to other CCR3 ligands.

Northern blot analysis demonstrates that Eotaxin-3 mRNA is constitutively expressed within the heart, liver, and ovary (1, 2). Low levels of Eotaxin-3 expression can also be detected in a variety of other tissues (1). Expression of Eotaxin-3 mRNA in vascular endothelial cells can be up-regulated by the cytokines IL-13 and IL-4 in a STAT6-dependent fashion (3, 7). In contrast to other potent eosinophil chemoattractants (*i.e.* Eotaxin-1, RANTES and MCP-4) that are induced by proinflammatory cytokines, neither TNF-α, IL-1β, IFN-γ, nor TNF-α plus IFN-γ are effective at up-regulating Eotaxin-3 mRNA expression.

Eotaxin-3 appears to be important for contributing to eosinophil accumulation in atopic disease. The mRNA expression of Eotaxin-3, unlike Eotaxin-1 or Eotaxin-2, is up-regulated in asthmatics in the stages following allergen challenge (8). These data suggest the possibility that Eotaxin-3 may be responsible for the continuing recruitment of eosinophils to asthmatic airways during this period.

The Quantikine Human CCL26/Eotaxin-3 Immunoassay is a 4.5 hour solid phase ELISA designed to measure Eotaxin-3 levels in cell culture supernates, serum, and plasma. It contains *E. coli*-expressed, recombinant human Eotaxin-3 and antibodies raised against the recombinant factor. Results obtained for naturally occuring human Eotaxin-3 showed linear curves that were parallel to the standard curves obtained using the kit standards. These results indicate that this kit can be used to determine relative mass values for natural human Eotaxin-3.

PRINCIPLE OF THE ASSAY

This assay employs the quantitative sandwich enzyme immunoassay technique. A monoclonal antibody specific for human Eotaxin-3 has been pre-coated onto a microplate. Standards and samples are pipetted into the wells and any Eotaxin-3 present is bound by the immobilized antibody. After washing away any unbound substances, an enzyme-linked monoclonal antibody specific for human Eotaxin-3 is added to the wells. Following a wash to remove any unbound antibody-enzyme reagent, a substrate solution is added to the wells and color develops in proportion to the amount of Eotaxin-3 bound in the initial step. The color development is stopped and the intensity of the color is measured.

LIMITATIONS OF THE PROCEDURE

- FOR RESEARCH USE ONLY. NOT FOR USE IN DIAGNOSTIC PROCEDURES.
- The kit should not be used beyond the expiration date on the kit label.
- Do not mix or substitute reagents with those from other lots or sources.
- If samples generate values higher than the highest standard, dilute the samples with Calibrator Diluent and repeat the assay.
- Any variation in standard diluent, operator, pipetting technique, washing technique, incubation time or temperature, and kit age can cause variation in binding.
- Variations in sample collection, processing, and storage may cause sample value differences.
- This assay is designed to eliminate interference by other factors present in biological samples. Until all factors have been tested in the Quantikine Immunoassay, the possibility of interference cannot be excluded.

TECHNICAL HINTS

- When mixing or reconstituting protein solutions, always avoid foaming.
- To avoid cross-contamination, change pipette tips between additions of each standard level, between sample additions, and between reagent additions. Also, use separate reservoirs for each reagent.
- To ensure accurate results, proper adhesion of plate sealers during incubation steps is necessary.
- When using an automated plate washer, adding a 30 second soak period following the addition of Wash Buffer, and/or rotating the plate 180 degrees between wash steps may improve assay precision.
- Substrate Solution should remain colorless until added to the plate. Keep Substrate Solution protected from light. Substrate Solution should change from colorless to gradations of blue.
- Stop Solution should be added to the plate in the same order as the Substrate Solution. The color developed in the wells will turn from blue to yellow upon addition of the Stop Solution. Wells that are green in color indicate that the Stop Solution has not mixed thoroughly with the Substrate Solution.

MATERIALS PROVIDED & STORAGE CONDITIONS

Store the unopened kit at 2-8 °C. Do not use past kit expiration date.

PART	PART #	DESCRIPTION	STORAGE OF OPENED/ RECONSTITUTED MATERIAL
Human Eotaxin-3 Microplate	891106	96 well polystyrene microplate (12 strips of 8 wells) coated with a monoclonal antibody specific for human Eotaxin-3.	Return unused wells to the foil pouch containing the desiccant pack. Reseal along entire edge of the zip-seal. May be stored for up to 1 month at 2-8 °C.*
Human Eotaxin-3 Conjugate	891107	21 mL of a monoclonal antibody specific for human Eotaxin-3 conjugated to horseradish peroxidase with preservative.	May be stored for up to 1 month at 2-8 °C.*
Human Eotaxin-3 Standard	891108	Recombinant human Eotaxin-3 in a buffered protein base with preservative; lyophilized. *Refer to the vial label for reconstitution volume.*	
Assay Diluent RD1S	895137	11 mL of a buffered protein base with preservatives.	
Calibrator Diluent RD5V	895425	21 mL of a buffered protein base with preservatives.	
Wash Buffer Concentrate	895003	21 mL of a 25-fold concentrated solution of buffered surfactant with preservative. *May turn yellow over time.*	
Color Reagent A	895000	12 mL of stabilized hydrogen peroxide.	
Color Reagent B	895001	12 mL of stabilized chromogen (tetramethylbenzidine).	
Stop Solution	895032	6 mL of 2 N sulfuric acid.	
Plate Sealers	N/A	4 adhesive strips.	

* Provided this is within the expiration date of the kit.

OTHER SUPPLIES REQUIRED

- Microplate reader capable of measuring absorbance at 450 nm, with the correction wavelength set at 540 nm or 570 nm.
- Pipettes and pipette tips.
- Deionized or distilled water.
- Squirt bottle, manifold dispenser, or automated microplate washer.
- 500 mL graduated cylinder.
- Horizontal orbital microplate shaker (0.12" orbit) capable of maintaining a speed of 500 ± 50 rpm.
- Test tubes for dilution of standards.
- Human Eotaxin-3 Controls (optional; R&D Systems, Catalog # QC22).

PRECAUTIONS

The Stop Solution provided with this kit is an acid solution.

Some components in this kit contain a preservative which may cause an allergic skin reaction. Avoid breathing mist.

Color Reagent B may cause skin, eye, and respiratory irritation. Avoid breathing fumes.

Wear protective gloves, clothing, eye, and face protection. Wash hands thoroughly after handling. Please refer to the MSDS on our website prior to use.

SAMPLE COLLECTION & STORAGE

The sample collection and storage conditions listed below are intended as general guidelines. Sample stability has not been evaluated.

Cell Culture Supernates - Remove particulates by centrifugation and assay immediately or aliquot and store samples at ≤ -20 °C. Avoid repeated freeze-thaw cycles.

Serum - Use a serum separator tube (SST) and allow samples to clot for 30 minutes at room temperature before centrifugation for 15 minutes at 1000 x g. Remove serum and assay immediately or aliquot and store samples at ≤ -20 °C. Avoid repeated freeze-thaw cycles.

Plasma - Collect plasma using heparin or EDTA as an anticoagulant. Centrifuge for 15 minutes at 1000 x g within 30 minutes of collection. Assay immediately or aliquot and store samples at ≤ -20 °C. Avoid repeated freeze-thaw cycles.

Note: *Citrate plasma has not been validated for use in this assay.*

REAGENT PREPARATION

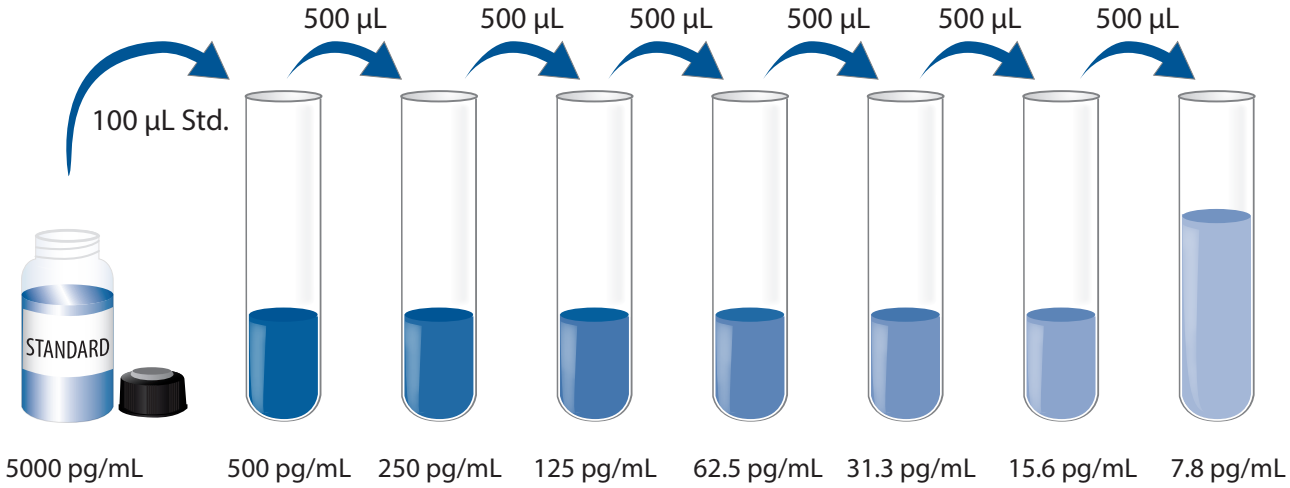
Bring all reagents to room temperature before use.

Wash Buffer - If crystals have formed in the concentrate, warm to room temperature and mix gently until the crystals have completely dissolved. Add 20 mL of Wash Buffer Concentrate to deionized or distilled water to prepare 500 mL of Wash Buffer.

Substrate Solution - Color Reagents A and B should be mixed together in equal volumes within 15 minutes of use. Protect from light. 200 µL of the resultant mixture is required per well.

Human Eotaxin-3 Standard - **Refer to the vial label for reconstitution volume.** Reconstitute the Human Eotaxin-3 Standard with deionized or distilled water. This reconstitution produces a stock solution of 5,000 pg/mL. Allow the standard to sit for a minimum of 15 minutes with gentle agitation prior to making dilutions.

Pipette 900 µL of Calibrator Diluent RD5V into the 500 pg/mL tube. Pipette 500 µL into the remaining tubes. Use the stock solution to produce a dilution series (below). Mix each tube thoroughly before the next transfer. The 500 pg/mL standard serves as the high standard. Calibrator Diluent RD5V serves as the zero standard (0 pg/mL).



ASSAY PROCEDURE

Bring all reagents and samples to room temperature before use. It is recommended that all standards, samples, and controls be assayed in duplicate.

1. Prepare all reagents, working standards, and samples as directed in the previous sections.
2. Remove excess microplate strips from the plate frame, return them to the foil pouch containing the desiccant pack, and reseal.
3. Add 100 µL of Assay Diluent RD1S to each well.
4. Add 100 µL of Standard, control, or sample per well. Cover with the adhesive strip provided. Incubate for 2 hours at room temperature on a horizontal orbital microplate shaker (0.12" orbit) set at 500 ± 50 rpm.
5. Aspirate each well and wash, repeating the process three times for a total of four washes. Wash by filling each well with Wash Buffer (400 µL) using a squirt bottle, manifold dispenser, or autowasher. Complete removal of liquid at each step is essential to good performance. After the last wash, remove any remaining Wash Buffer by aspirating or decanting. Invert the plate and blot it against clean paper towels.
6. Add 200 µL of Human Eotaxin-3 Conjugate to each well. Cover with a new adhesive strip. Incubate for 2 hours at room temperature on the shaker.
7. Repeat the aspiration/wash as in step 5.
8. Add 200 µL of Substrate Solution to each well. Incubate for 30 minutes at room temperature **on the benchtop. Protect from light.**
9. Add 50 µL of Stop Solution to each well. The color in the wells should change from blue to yellow. If the color in the wells is green or if the color change does not appear uniform, gently tap the plate to ensure thorough mixing.
10. Determine the optical density of each well within 30 minutes, using a microplate reader set to 450 nm. If wavelength correction is available, set to 540 nm or 570 nm. If wavelength correction is not available, subtract readings at 540 nm or 570 nm from the readings at 450 nm. This subtraction will correct for optical imperfections in the plate. Readings made directly at 450 nm without correction may be higher and less accurate.

CALCULATION OF RESULTS

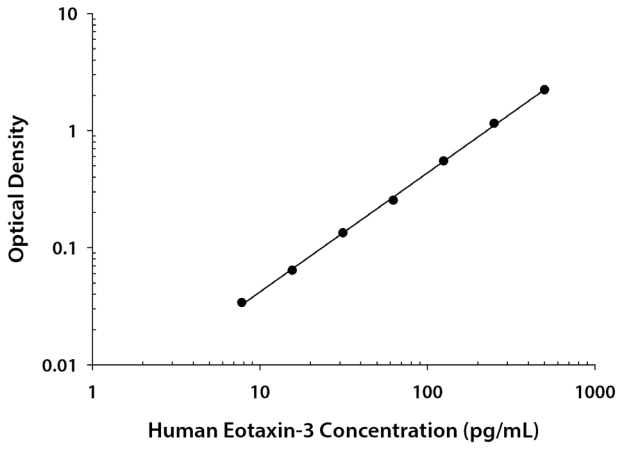
Average the duplicate readings for each standard, control, and sample and subtract the average zero standard optical density (O.D.).

Create a standard curve by reducing the data using computer software capable of generating a log/log curve-fit. As an alternative, construct a standard curve by plotting the mean absorbance for each standard on the y-axis against the concentration on the x-axis and draw a best fit curve through the points on a log/log graph. The data may be linearized by plotting the log of the human Eotaxin-3 concentrations versus the log of the O.D. on a linear scale, and the best fit line can be determined by regression analysis.

If the samples have been diluted, the concentration read from the standard curve must be multiplied by the dilution factor.

TYPICAL DATA

This standard curve is provided for demonstration only. A standard curve should be generated for each set of samples assayed.



(pg/mL)	O.D.	Average	Corrected
0	0.015 0.012	0.014	—
7.8	0.050 0.045	0.048	0.034
15.6	0.082 0.077	0.078	0.064
31.3	0.152 0.144	0.148	0.134
62.5	0.278 0.266	0.268	0.254
125	0.579 0.548	0.564	0.550
250	1.155 1.177	1.166	1.152
500	2.296 2.192	2.244	2.230

PRECISION

Intra-assay Precision (Precision within an assay)
Three samples of known concentration were tested twenty times on one plate to assess intra-assay precision.

Inter-assay Precision (Precision between assays)
Three samples of known concentration were tested in forty separate assays to assess inter-assay precision. Assays were performed by at least three technicians using two lots of components.

	Intra-Assay Precision			Inter-Assay Precision		
Sample	1	2	3	1	2	3
n	20	20	20	40	40	40
Mean (pg/mL)	42.4	114	291	50.3	126	293
Standard deviation	2.37	4.23	9.62	3.78	5.53	18.8
CV (%)	5.6	3.7	3.3	7.5	4.4	6.4

RECOVERY

The recovery of human Eotaxin-3 spiked to levels throughout the range of the assay in various matrices was evaluated.

Sample Type	Average % Recovery	Range
Cell culture media (n=4)	100	92-112%
Serum (n=5)	91	86-98%
EDTA plasma (n=5)	92	85-101%
Heparin plasma (n=5)	92	86-100%

LINEARITY

To assess the linearity of the assay, samples spiked with high concentrations of human Eotaxin-3 were serially diluted with Calibrator Diluent to produce samples with values within the dynamic range of the assay.

		Cell culture media (n=4)	Serum (n=5)	EDTA plasma (n=5)	Heparin plasma (n=5)
1:2	Average % of Expected	97	103	101	97
	Range (%)	96-99	99-106	95-110	93-104
1:4	Average % of Expected	100	103	104	98
	Range (%)	93-105	99-107	98-112	94-104
1:8	Average % of Expected	99	105	106	106
	Range (%)	89-108	101-110	96-115	101-108
1:16	Average % of Expected	104	106	109	108
	Range (%)	90-112	99-111	106-114	101-114

SENSITIVITY

Thirty-nine assays were evaluated and the minimum detectable dose (MDD) of human Eotaxin-3 ranged from 0.87-5.2 pg/mL. The mean MDD was 2.33 pg/mL.

The MDD was determined by adding two standard deviations to the mean optical density value of twenty zero standard replicates and calculating the corresponding concentration.

CALIBRATION

This immunoassay is calibrated against highly purified *E. coli*-expressed recombinant human Eotaxin-3 produced at R&D Systems.

SAMPLE VALUES

Serum/Plasma - Samples from apparently healthy volunteers were evaluated for the presence of human Eotaxin-3 in this assay. No medical histories were available for the donors used in this study.

Sample Type	Mean of Detectable (pg/mL)	% Detectable	Range (pg/mL)
Serum (n=36)	9.7	8	ND-10.3
EDTA plasma (n=36)	12.7	6	ND-14.7

ND=Non-detectable

Sample Type	Mean (pg/mL)	Range (pg/mL)	Standard Deviation (pg/mL)
Heparin plasma (n=61)	50.8	17-146	23.8

Cell Culture Supernates - HUVEC human umbilical vein endothelial cells (5 x 10^6 cells/mL) were cultured in EGM supplemented with 2% fetal calf serum, 2 mM L-glutamine, 100 U/mL penicillin, 100 µg/mL streptomycin sulfate and bovine brain extract. Cells were grown to confluence, trypsinized, and the supernate was poured off and assayed. Cells were cultured unstimulated or stimulated with 100 ng/mL of recombinant human IL-4 for 24 hours. Aliquots of the cell culture supernates were removed and assayed for human Eotaxin-3.

Condition	Day 1 (pg/mL)
Unstimulated	ND
Stimulated	9500

ND=Non-detectable

SPECIFICITY

This assay recognizes natural and recombinant human Eotaxin-3.

The factors listed below were prepared at 50 ng/mL in Calibrator Diluent and assayed for cross-reactivity. Preparations of the following factors at 50 ng/mL in a mid-range recombinant human Eotaxin-3 control were assayed for interference. No significant cross-reactivity or interference was observed.

Recombinant human:

6Ckine
CTACK
Eotaxin
Eotaxin-2
HCC-1
HCC-4
I-309
MCP-1
MCP-2
MCP-3
MCP-4
MDC
MIP-1α
MIP-1β
MIP-1δ
MIP-3α
MIP-3β
MPIF-1
PARC
RANTES
TARC
TECK

Recombinant mouse:

6Ckine
CTACK
Eotaxin
JE/MCP-1
MARC
MCP-5
MDC
MIP-1α
MIP-1β
MIP-3α
MIP-3β
RANTES
TARC
TECK
TCA-3

Recombinant rat:

MIP-3α

A splice variant of recombinant human Eotaxin-3 with 68 amino acids as compared to 71 amino acids, was tested for cross-reactivity. The highest cross-reactivity observed was 15.8% at a concentration of 3 ng/mL.

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