

**COMPONENTS**

Kit Component	Amount
96-well plate pre-coated with anti-human Eotaxin antibody	1 Plate
Protein Standard: Lyophilized recombinant human Eotaxin	2 tubes, 10 ng/tube
Sample Diluent Buffer	30 ml
Biotinylated Antibody (Anti-human Eotaxin)	130 µl (100x)
Antibody Diluent Buffer	12ml
Avidin-Biotin-Peroxidase Complex (ABC) Solution	130 µl (100x)
ABC Diluent Buffer	12 ml
Tetramethylbenzidine (TMB) Color Developing Agent	10 ml
TMB Stop Solution	10 ml

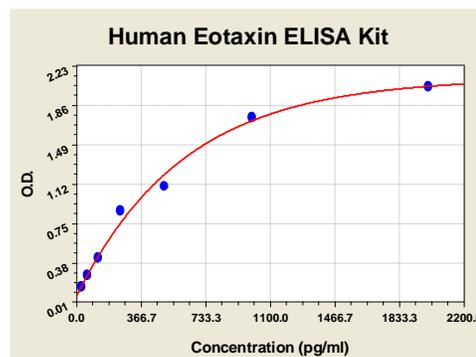
**Washing Buffer (not provided): TBS or PBS**

0.01M TBS: Add 1.2g Tris, 8.5g NaCl; 450µl of purified acetic acid or 700µl of concentrated hydrochloric acid to 900ml H<sub>2</sub>O and adjust pH to 7.2-7.6. Adjust the total volume to 1L.

0.01M PBS: Add 8.5g sodium chloride, 1.4g Na<sub>2</sub>HPO<sub>4</sub> and 0.2g NaH<sub>2</sub>PO<sub>4</sub> to 900ml distilled H<sub>2</sub>O and adjust pH to 7.2-7.6. Adjust the total volume to 1L.

**Storage**

Store at 4°C. Cell Applications, Inc. recommends using the kit within 6 months of order.



X	pg/ml	0.0	31.2	62.5	125	250	500	1000	2000
Y	O.D.450	0.050	0.151	0.260	0.426	0.871	1.097	1.749	2.034

**Figure 1: Eotaxin Standard Curve.** Using the Human Eotaxin ELISA Kit, O.D. data was graphed against Eotaxin protein concentration. The TMB reaction was incubated at 37°C for 15 min.

**BACKGROUND**

The c-erbB family of receptors includes four distinct receptors, namely c-erb B1 (EGF receptor), 2, 3 and 4 (HER1, 2, 3 and 4, respectively). The epidermal growth factor (EGF) and related peptides bind the ErbB receptors, inducing the formation of different homo- and heterodimers. Receptor dimerization promotes activation of the intrinsic kinase, leading to phosphorylation of specific tyrosines located in the ErbB's cytoplasmic region. These phosphorylated residues serve as docking sites for a variety of signaling molecules whose recruitment stimulates intracellular signaling cascades, which ultimately control diverse genetic programs.<sup>1</sup> In contrast to other receptor tyrosine kinases, the kinase domain of EGFR (ERBB1) does not require phosphorylation for activation. Consequently, the overall activation state of the receptor is controlled by constant balancing of activity favoring and activity suppressing actions within the receptor molecule. Influences of the membrane microenvironment and context dependent interactions with varying sets of signaling partners are superimposed on this system of intramolecular checks and balances.<sup>2</sup>

It was shown that EGFR overexpression and mutations occurred in many tumors. The EGFR is known to be involved in carcinogenic processes such as cell proliferation, apoptosis, angiogenesis, cell motility, and metastasis. Preclinical and clinical studies have shown that targeting EGFR is a valid method for anticancer therapy.<sup>3</sup> Strategies aimed at inhibiting the EGFR pathway included different classes of compounds, with monoclonal antibodies and tyrosine kinase inhibitors being the most widely-investigated agents in cancer therapy.

**References**

- Holbro, T. & Hynes, N. E.: Annu Rev Pharmacol Toxicol. 44:195, 2004.
- Warren, C.M. & Landgraf, R.: Cell Signal 18:923, 2006.
- Arteaga, C. L.: Semin Oncol. 29(5 Suppl 14):3, 2002.

**ELISA OVERVIEW**

Cell Applications ELISA Kits are based on standard sandwich enzyme-linked immunosorbent assay technology. Freshly prepared standards, samples, and solutions are recommended for best results.

1. Prepare test samples.
2. Prepare a protein standard of the target protein.
3. Add test samples and standards to the pre-coated 96-well plate. Do not wash.
4. Add biotinylated detection antibodies. Wash.
5. Add Avidin-Biotin-Peroxidase Complex (ABC) Solution. Wash.
6. Add Tetramethylbenzidine (TMB) Color Developing Agent, containing HRP substrate.
7. Add TMB Stop Solution
8. Subject the plate to analysis.

**NOTES:**

- Before using the kit, quick spin tubes to bring down all solution to the bottom of tube.
- Duplicate assay wells are recommended for both standard and sample testing.
- Do not let the 96-well plate dry, this will lead to inactivation of plate components.
- When diluting samples and reagents, ensure that they are mixed completely and evenly.
- Pre-warm diluted ABC and TMB solutions at 37°C for 30 min before use to avoid variable temperature effects.
- For washes, use TBS or PBS. Do not touch well walls.
- A protein standard is included in the kit. A protein standard detection curve should be generated with each experiment, no more than 2 hours prior to the experiment.
- The user will determine sample dilution fold by estimation of target protein amount in samples.

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## PROTOCOL

### I. Plate Washing

Discard the solution in the plate without touching the side walls. Blot the plate onto paper towels or other absorbent material. Soak each well with at least 0.3 ml PBS or TBS buffer for 1–2 minutes. Repeat this process two additional times for a total of three washes.

### II. Preparation of Test Samples

#### Test Sample Processing

- **Cell culture supernate, tissue lysate or body fluids:** Remove particulates by centrifugation.
- **Serum:** Allow the serum to clot in a serum separator tube (about 2 hours) at room temperature. Centrifuge at approximately 1000 X g for 10 min.
- **Plasma:** Collect plasma using heparin, EDTA, citrate as an anticoagulant. Centrifuge for 15 min at 1000 x g within 30 min of collection. Analyze immediately or aliquot and store frozen at -20°C.

#### Sample Dilution Guideline

Estimate the concentration of the target protein in the sample and select a proper dilution factor such that the diluted target protein concentration falls within the standard curve range. Depending on the sample, several trial dilutions may be necessary. Dilute the sample using the provided diluent buffer, mixing well. Suggested working dilutions of samples are as follows:

Target Protein Concentration Range	Sample Working Dilution	Sample Vol.	Diluent Buffer Vol.
20-200 ng/ml	1:100	1 µl	99 µl
2-20 ng/ml	1:10	10 µl	90 µl
31.2-2000 pg/ml	1:2	50 µl	50 µl
≤31.2 pg/ml	n/a	100µl	n/a

If samples will be assayed within 24 hours, store at 2-8°C. For long-term storage, aliquot and freeze samples at -20°C. Avoid repeated freeze-thaw cycles.

### III. Preparation of Reagents

#### Reconstitution of the Standard

The standard solutions should be prepared no more than 2 hours prior to the experiment. Two tubes of the standard are included in each kit. Use one tube for each experiment.

1. 10,000pg/ml of human Eotaxin standard solution: Add 1 ml sample diluent buffer into one tube, keep the tube at room temperature for 10 min and mix thoroughly.
2. 2000pg/ml of human Eotaxin standard solution: Add 0.2 ml of the above 10ng/ml Eotaxin standard solution into 0.8 ml sample diluent buffer and mix thoroughly.
3. 1000pg/ml→31.2pg/ml of human Eotaxin standard solutions: Label 6 Eppendorf tubes with 1000pg/ml, 500pg/ml, 250pg/ml, 125pg/ml, 62.5pg/ml, 31.2pg/ml, respectively. Aliquot 0.3 ml of the sample diluent buffer into each tube. Add 0.3 ml of the above 2000pg/ml Eotaxin standard solution into 1st tube and mix. Transfer 0.3 ml from 1st tube to 2nd tube and mix. Transfer 0.3 ml from 2nd tube to 3rd tube and mix, and so on.

#### Preparation of Biotinylated Antibody Working Solution

The solution should be prepared no more than 2 hours prior to the experiment.

1. The total volume should be: 0.1ml/well x (the number of wells). (Allowing 0.1-0.2 ml more than total volume)
2. Biotinylated antibody should be diluted in 1:100 with the antibody diluent buffer and mixed thoroughly.

### Preparation of the Avidin-Biotin-Peroxidase Complex (ABC) Working Solution

The solution should be prepared no more than 1 hour prior to the experiment.

1. The total volume should be: 0.1ml/well x (the number of wells). (Allowing 0.1-0.2 ml more than total volume)
2. Avidin- Biotin-Peroxidase Complex (ABC) should be diluted in 1:100 with the ABC dilution buffer and mixed thoroughly.

### IV. ELISA

The ABC working solution and TMB color developing agent must be kept warm at 37°C for 30 min before use. When diluting samples and reagents, they must be mixed completely and evenly. A standard detection curve should be prepared for each experiment. The user will decide sample dilution fold by crude estimation of target protein amount in samples.

1. Aliquot 0.1ml per well of the 2000pg/ml, 1000pg/ml, 500pg/ml, 250pg/ml, 125pg/ml, 62.5pg/ml, 31.2pg/ml human Eotaxin standard solutions into the precoated 96-well plate. Add 0.1ml of the sample diluent buffer into the control well (Blank well). Add 0.1ml of each properly diluted sample of human sera, plasma, body fluids, tissue lysates or cell culture supernatants to each empty well. See “Sample Dilution Guideline” for details. We recommend that each human Eotaxin standard solution and each sample is measured in duplicate.
2. Seal the plate with the cover and incubate at 37°C for 90 min.
3. Remove the cover, discard plate content, and blot the plate onto paper towels or other absorbent material. Do NOT let the wells completely dry at any time.
4. Add 0.1ml of biotinylated anti-human Eotaxin antibody working solution into each well and incubate the plate at 37°C for 60 min.
5. Wash the plate three times with 0.01M TBS or 0.01M PBS, and each time let washing buffer stay in the wells for 1 min. Discard the washing buffer and blot the plate onto paper towels or other absorbent material.
6. Add 0.1ml of prepared ABC working solution into each well and incubate the plate at 37°C for 30 min.
7. Wash plate 5 times with 0.01M TBS or 0.01M PBS, and each time let washing buffer stay in the wells for 1-2 min. Discard the washing buffer and blot the plate onto paper towels or other absorbent material.
8. Add 90 µl of prepared TMB color developing agent into each well and incubate plate at 37°C for 7-10 min (shades of blue can be seen in the wells with the four most concentrated human Eotaxin standard solutions; the other wells show no obvious color).
9. Add 0.1ml of prepared TMB stop solution into each well. The color changes into yellow immediately.
10. Read the O.D. absorbance at 450nm in a microplate reader within 30 min after adding the stop solution.

### V. Calculating Protein Concentration

- For all wells, determine O.D.450(Relative):  
 $O.D.450(Relative) = O.D.450(Reading) - O.D.450(Blank)$
- Plot the standard curve:  
Plot O.D.450(Relative) of each standard solution (Y) vs. the respective concentration of the standard solution (X). See **Figure 1** for a typical standard curve.
- The target protein concentration in samples can be interpolated from the standard curve. Multiply the interpolated concentration by the dilution factor to obtain the target protein concentration in the sample.

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