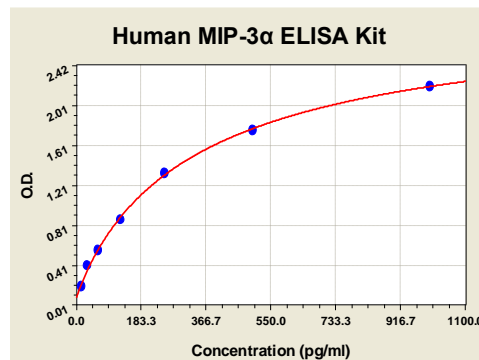


KIT COMPONENTS

Component	Amount
96-well plate pre-coated with anti-human MIP-3alpha antibody	1 Plate
Protein Standard: Lyophilized recombinant human MIP-3alpha	2 tubes, 10 ng/tube
Sample Diluent Buffer	30 ml
Biotinylated Antibody (Anti-human MIP-3alpha)	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

Storage

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



X	pg/ml	0.0	15.6	31.3	62.5	125	250	500	1000	
Y	O.D.	0.450	0.085	0.201	0.408	0.566	0.869	1.339	1.770	2.204

Figure 1: MIP-3alpha Standard Curve. Using the human MIP-3alpha ELISA Kit, O.D. data was graphed against MIP-3alpha protein concentration. The TMB reaction was incubated at 37° C for 16 min.

BACKGROUND

MIP-3alpha was identified by several groups as a result of large scale sequencing projects and alternatively designated LARC, Exodus, ckbeta4, and CCL20. MIP-3alpha is not very closely related to other CC chemokines, with 20–28% amino acid sequence identity with other chemokines, and, correspondingly, the MIP-3alpha gene is not in the major CC chemokine gene cluster at 17q11.2 but is located instead at 2q33-q37. Northern blotting and analysis of cDNA libraries revealed expression of the MIP-3alpha gene in lung, liver, and lymphoid tissue, and in activated monocytes, endothelial cells, dendritic cells, fibroblasts, and T cells. Immature dendritic cells (DCs) express CCR6, the only receptor for MIP-3alpha.¹ MIP-3alpha triggers adaptive immunity by attracting immature DCs to the site of inflammation. Immature DCs also express CCR1, CCR4, and CCR5, which account for chemotactic response to MIP-1alpha (CCL3) and CCL5. After antigen uptake, immature DCs lose their responsiveness to MIP-1alpha and MIP-3alpha, and CCR7 expression is strongly up-regulated. Mature DCs migrate to lymph nodes in response to MIP-3beta (CCL19), a ligand of CCR7. MIP-1alpha, MIP-3alpha, and MIP-3beta play critical roles in the regulation of DC trafficking. Besides its activity on dendritic cells, MIP-3alpha has been found to be chemotactic for freshly isolated T cells, to be able to inhibit colony formation by hematopoietic progenitors *in vitro*, and to be able to trigger adhesion of memory CD4+ T cells to ICAM-1-coated glass. In addition, it was shown that MIP-3alpha possesses antibacterial activity of greater potency than human beta-Defensin-1 and beta-Defensin-2 against *E. coli* and *Staphylococcus aureus*.² Moreover, it was reported that MIP-3alpha has been shown to promote tumor cell migration by upregulation of MMPs.³

Reference

- Liao, F. et al: J. Immunol. 162: 186-94, 1999
- Hoover, D.M. et al: J. Biol. Chem. 277:37647-54,2002
- Campbell, A.S. et al: J. Surg. Res. 114:250-1, 2003

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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ELISA PROTOCOL

Preparation of Test Samples

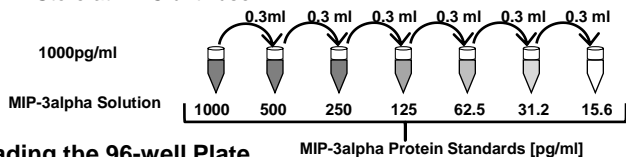
- Process Test Samples in the following manner:
 - Cell culture supernate, tissue lysate or body fluids:** Remove particulates by centrifugation. analyze immediately or aliquot and store at -20° C.
 - Serum:** Allow the serum to clot in a serum separator tube (about 30 min) at room temperature. Centrifuge at approximately 1000 x g for 15 min. Analyze the serum immediately or aliquot and store frozen at -20° C.
 - 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..
- 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 15.6-1000 pg/ml 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.
10-100 ng/ml	1:100	1 µl	99 µl
1-10 ng/ml	1:10	10 µl	90 µl
15.6-1000 pg/ml	1:2	50 µl	50 µl
≤15.6 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.

Preparation of Standard Solutions (15.6-1000 pg/ml)

- Reconstitute the Lypophilized Recombinant Protein to make a 10,000 pg/ml human MIP-3alpha solution. Add 1 ml Sample Diluent Buffer to a tube of lypophilized protein, keep the tube at room temperature for 10 min. Mix thoroughly.
- Add 0.1 ml of the mixed 10,000 pg/ml MIP-3alpha solution to the eppendorf tube containing 0.9 ml diluent buffer and mix to make a 1000 pg/ml MIP-3alpha solution.
- Label 6 eppendorf tubes with the human MIP-3alpha protein concentrations to be prepared by serial dilution :500pg/ml, 250pg/ml, 125pg/ml, 62.5pg/ml, 31.3pg/ml, 15.6pg/ml
- Aliquot 0.3 ml of the Sample Diluent Buffer to the labeled tubes.
- Serially dilute the protein standards into their respectively labeled tubes. Transfer 0.3 ml from the 1000pg/ml MIP-3alpha Solution to the 500pg/ml eppendorf tube and mix thoroughly. Transfer 0.3 ml of the 500 pg/ml solution to the 250pg/ml tube and mix thoroughly, Transfer 0.3 ml of the 250pg/ml solution to the 125pg/ml tube and mix, and so on to make the 62.5, 31.3 and 15.6 pg/ml solutions.
- Store at 4° C until use.



Loading the 96-well Plate

- Aliquot 0.1 ml of the sample diluent buffer into a control well to serve as the Blank. This will yield the O.D.450(Blank) reading.
- Aliquot 0.1 ml of the standard solutions of the **Preparation of Standard Solutions** (15.6-1000pg/ml) into empty wells of the precoated 96-well plate. Duplicate measurements of standards are recommended.

- Aliquot 0.1 ml of each properly diluted test sample to empty wells prepared in **Step 2**. Duplicate measurements of each test sample are recommended.
- Cover the 96-well plate and incubate at 37° C for 90 min.
- During the **Step 13** incubation period, prepare a stock of Biotinylated 1:100 Antibody Working Solution. Count the number of reactions and multiply by 0.1 ml/well for the Working Solution total volume (preparation of 1-2 reactions in excess of the number of wells is recommended). Dilute the Biotinylated Antibody to 1:100 in Antibody Diluent Buffer and mix thoroughly. Use the working solution within 2 hours.
- Upon completion of the 90 min incubation of **Step 13**, remove the cover of the 96 well plate and discard plate well contents. Blot the plate onto paper towels or other absorbent material. DO NOT let the wells completely dry at any time.
- Add 0.1 ml of the Biotinylated 1:100 Antibody Working Solution (prepared in **Step 14**) to each well and incubate the plate at 37° C for 60 min.
- During the incubation period of **Step 16**, prepare a stock of ABC Working Solution. Count the number of reactions and multiply by 0.1 ml/well for the Working Solution total volume (preparation of 1-2 reactions in excess of the number of wells is recommended). Dilute the ABC Stock Solution to 1:100 in ABC Diluent Buffer and mix thoroughly. Pre-warm the ABC working solution at 37° C for 30 min before use. Use the working solution within 1 hour.
- Upon completion of the 60 min incubation of **Step 16**, wash the plate 3 times with 0.3 ml TBS or PBS. For each wash, leave washing buffer in the wells for 1-2 min. Discard the washing buffer and blot the plate onto paper towels or other absorbent material.
- Add 0.1 ml of prepared ABC Working Solution (prepared in **Step 17**) to each well and incubate the plate at 37° C for 30 min.
- Add 90 µl of the pre-warmed TMB Color Developing Agent into each well and incubate at 37° C for 10-15 min (shades of blue can be seen in the wells with the four most concentrated Protein Standard Solutions; the other control wells should show no obvious color).
- During the incubation period of **Step 19**, pre-warm TMB Color Developing Agent at 37° C for 30 min before use.
- Upon completion of the 30 min incubation of **Step 19**, wash the plate 5 times with 0.3 ml TBS or PBS. For each wash, leave the washing buffer in the wells for 1-2 min. Discard the washing buffer and blot the plate onto paper towels or other absorbent material.
- Add 0.1 ml of the TMB Stop Solution to each well. The acidic stop solution will change the mixture color to yellow. The yellow intensity is proportional to the amount of target protein captured by the plate.
- Read the O.D. absorbance at 450nm in a microplate reader within 30 min after adding the stop solution. These readings are the O.D.450(Reading).

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 human MIP-3alpha concentration of the 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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