**Bethyl Laboratories, Inc., www.fortislife.com**

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Datasheet/User Guide

# Mouse TNF-alpha ELISA Kit

Catalog Number: E92-009

Lot Number:  250811

For Serum, Plasma, Cell Culture Supernatants

## Introduction

Tumor necrosis factor alpha (TNF-alpha), also known as lymphotoxin and cachectin, is a pleiotropic cytokine. TNF alpha is a smaller cytokine with a molecular weight of 26 kDa (transmembrane) and 17 kDa (soluble) that binds to the same receptors producing a vast array of effects similar to those of TNF-beta. Both TNF-alpha and TNF-beta share 30% amino acid homology and have similar biological activities. TNF-alpha is produced primarily by monocytes, neutrophils and macrophages. It is found in synovial cells and macrophages in the tissues. TNF-alpha and TNF-beta can modulate many immune and inflammatory functions, while having the ability to inhibit tumor growth. Target tumor cells must express TNF receptors 1 (55 kDa) and 2 (75 kDa) to be killed, with the p55 receptor mediating the cytotoxic response.

The Mouse TNF-alpha ELISA kit is an in-vitro enzyme-linked immunosorbent assay for the quantitative measurement of Mouse TNF-alpha in serum, plasma (Mouse TNF-alpha concentration is low in normal serum/plasma and may not be detectable in this assay), and cell culture supernatants. This assay employs an antibody specific for Mouse TNF-alpha coated on a 96-well plate. Standards and samples are pipetted into the wells and TNF-alpha present in a sample is bound to the wells by the immobilized antibody. The wells are washed and biotinylated anti-Mouse TNF-alpha antibody is added. After washing away unbound biotinylated antibody, HRP-conjugated streptavidin is pipetted to the wells. The wells are again washed, a TMB substrate solution is added to the wells and color develops in proportion to the amount of TNF-alpha bound. The Stop Solution changes the color from blue to yellow, and the intensity of the color is measured at 450 nm.

## Storage/Shelf Life

2 - 8°C/6 months from date of receipt

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## Reagents

| Component | Size / Description | Storage / Stability After Preparation |
|---|---|---|
| Mouse TNF-alpha Microplate | 96 wells (12 strips x 8 wells) coated with anti-Mouse TNF-alpha. | 1 month at 4°C* |
| Mouse TNF-alpha Standard Protein | 2 vials of Mouse TNF-alpha. 1 vial is enough to run each standard in duplicate. | 1 week at -80°C |
| Mouse TNF-alpha Detection Antibody | 2 vials of biotinylated anti-Mouse TNF-alpha. Each vial is enough to assay half the microplate. | 5 days at 4°C |
| Wash Buffer | 25 ml of 20X concentrated solution. | 1 month at 4°C |
| HRP-Streptavidin | 200 µl 300X concentrated HRP-conjugated streptavidin. | Do not store and reuse. |
| TMB One-Step Substrate Reagent | 12 ml of 3,3,5,5'-tetramethylbenzidine (TMB) in buffer solution. | N/A |
| Stop Solution | 8 ml of 0.2 M sulfuric acid. | N/A |
| Assay Diluent A | 30 ml of diluent buffer, 0.09% sodium azide as preservative. | N/A |
| Assay Diluent B | 15 ml of 5X concentrated buffer. | 1 month at 4°C |

## Additional Materials Required (but not provided)

- Microplate reader capable of measuring absorbance at 450 nm.
- ELISA plate shaker or platform rocker.
- Precision pipettes to deliver 2 µl to 1 ml volumes.
- Adjustable 1-25 ml pipettes for reagent preparation.
- 100 ml and 1 liter graduated cylinders.
- Absorbent paper.
- Distilled or deionized water.
- Log-log graph paper or computer and software for ELISA data analysis.
- Tubes to prepare standard or sample dilutions.

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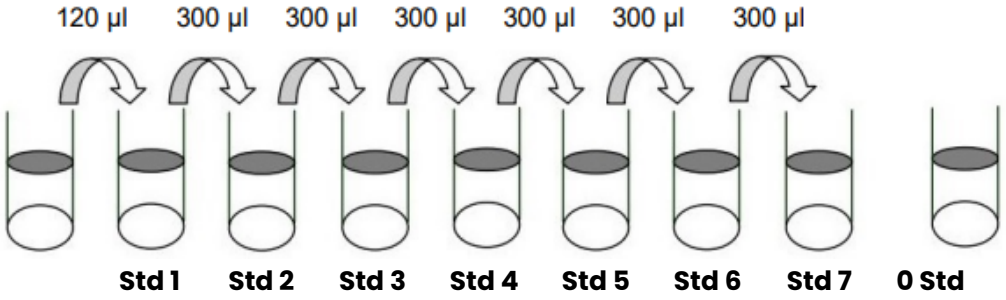
## Reagent Preparation

1. Bring all reagents and samples to room temperature before use.
2. Assay Diluent B should be diluted 5-fold with deionized or distilled water before use.
3. Sample dilution: Assay Diluent A should be used for dilution of serum and plasma samples. 1X Assay Diluent B should be used for dilution of cell culture supernatant samples. The suggested dilution for normal serum/plasma is 2-fold.

   Note: Levels of TNF-alpha may vary between different samples. Optimal dilution factors for each sample must be determined by the investigator.

4. Preparation of standard: Briefly spin a vial of standard protein. Add 400 µl Assay Diluent A (for serum/plasma samples) or 1X Assay Diluent B (for cell culture medium) into the Standard Protein vial to prepare a 50 ng/ml standard. Dissolve the powder thoroughly by a gentle mix. Add 120 µl TNF-alpha standard from the vial of Standard Protein, into a tube with 880 µl Assay Diluent A or 1X Assay Diluent B to prepare a 6,000 pg/ml stock standard solution. Pipette 300 µl Assay Diluent A or 1X Assay Diluent B into each tube. Use the stock standard solution to produce a dilution series (shown below). Mix each tube thoroughly before the next transfer. Assay Diluent A or 1X Assay Diluent B serves as the zero standard (0 pg/ml).




| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| **Diluent volume** | Std + 400 µl | 880 µl | 300 µl | 300 µl | 300 µl | 300 µl | 300 µl | 300 µl | 300 µl |
| **Concentration** | 50 ng/ml | 6000 pg/ml | 3000 pg/ml | 1500 pg/ml | 750 pg/ml | 375 pg/ml | 187.5 pg/ml | 93.75 pg/ml | 0 pg/ml |

5. If the Wash Buffer (20X) contains visible crystals, warm to room temperature and mix gently until dissolved. Dilute 25 ml of 20X Wash Buffer into 475 ml deionized or distilled water to yield 500 ml of 1X Wash Buffer.
6. Briefly spin the Detection Antibody vial before use. Add 100 µl of 1X Assay Diluent B into the vial to prepare a detection antibody concentrate. Pipette up and down to mix gently (the concentrate can be stored at 4ºC for 5 days). The detection antibody concentrate should be diluted 80-fold with 1X Assay Diluent B and used in step 5 Assay Procedure.
7. Briefly spin the HRP-Streptavidin concentrate vial and pipette up and down to mix gently before use, as precipitates may form during storage. HRP-Streptavidin concentrate should be diluted 300-fold with 1X Assay Diluent B. For example: Add 40 µl of HRP-Streptavidin concentrate into a tube with 12 ml 1X Assay Diluent B to prepare a final 300-fold diluted solution. Mix well. DO NOT STORE THE DILUTED SOLUTION FOR NEXT DAY USE.

## Assay Procedure

1. Bring all reagents and samples to room temperature before use. It is recommended that all standards and samples be run at least in duplicate.
2. Label removable 8-well strips as appropriate for your experiment.
3. Add 100 µl of each standard (see Reagent Preparation step 4) and sample into appropriate wells. Cover wells and incubate for 2.5 hours at room temperature with gentle shaking.
4. Discard the solution and wash 4 times with 1X Wash Solution. Wash by filling each well with Wash Buffer (300 µl) using a multi-channel Pipette 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.
5. Add 100 µl of 1X prepared biotinylated antibody (Reagent Preparation step 6) to each well. Incubate for 1 hour at room temperature with gentle shaking.
6. Discard the solution. Repeat the wash as in step 4.
7. Add 100 µl of prepared Streptavidin solution (see Reagent Preparation step 7) to each well. Incubate for 45 minutes at room temperature with gentle shaking.
8. Discard the solution. Repeat the wash as in step 4.
9. Add 100 µl of TMB One-Step Substrate Reagent to each well. Incubate for 30 minutes at room temperature in the dark with gentle shaking.
10. Add 50 µl of Stop Solution to each well. Read at 450 nm immediately.

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## Assay Procedure Summary

1. Prepare all reagents, samples and standards as instructed.
2. Add 100 µl standard or sample to each well. Incubate 2.5 hours at room temperature.
3. Add 100 µl prepared biotin antibody to each well. Incubate 1 hour at room temperature.
4. Add 100 µl prepared Streptavidin solution. Incubate 45 minutes at room temperature.
5. Add 100 µl TMB One-Step Substrate Reagent to each well. Incubate 30 minutes at room temperature.
6. Add 50 µl Stop Solution to each well. Read at 450 nm immediately.

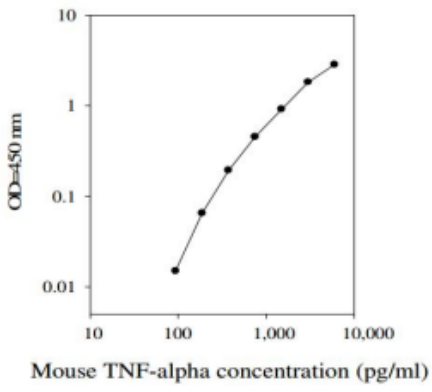
## Calculation of Results

Calculate the mean absorbance for each set of duplicate standards, controls and samples, and subtract the average zero standard optical density. Plot the standard curve on log-log graph paper or using Sigmaplot software, with standard concentration on the x-axis and absorbance on the y-axis. Draw the best-fit straight line through the standard points.

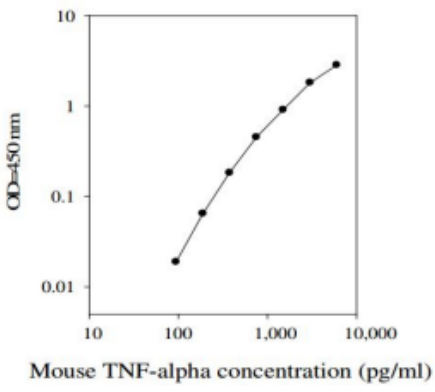
## Typical Data

The typical standard curve was generated using the Mouse TNF-alpha ELISA kit protocol. The standard curves are for demonstration only. A standard curve must be generated for each assay.

Assay Diluent A




Assay Diluent B




## Sensitivity

The minimum detectable dose of Mouse TNF-alpha was determined to be 60 pg/ml.

Minimum detectable dose is defined as the analyte concentration resulting in an absorbance that is 2 standard deviations higher than that of the blank (diluent buffer.)

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## Spiking & Recovery

Recovery was determined by spiking various levels of Mouse TNF-alpha into the sample types listed below. Mean recoveries are as follows:

| Sample Type | Average % Recovery | Range (%) |
|---|---|---|
| Serum | 94.2 | 82-103 |
| Plasma | 93.8 | 83-102 |
| Cell culture media | 92.5 | 84-104 |

## Linearity

| Sample Type | Serum | Plasma | Cell culture media |
|---|---|---|---|
| 1:2 dilution<br>Avg % of Expected<br>Range (%) | <br>93<br>84-102 | <br>94<br>83-103 | <br>92<br>82-103 |
| 1:4 dilution<br>Avg % of Expected<br>Range (%) | <br>94<br>82-103 | <br>95<br>84-104 | <br>96<br>83-104 |

## Reproducibility

Intra-Assay CV%: <10%

Inter-Assay CV%: <12%

## Specificity

This ELISA kit shows no cross-reactivity with any of the cytokines tested: Mouse CD30, L CD30, T CD40, CRG-2, CTACK, CXCL16, Eotaxin , Eotaxin-2, Fas Ligand, Fractalkine, GCSF, GM-CFS, IFN-gamma, IGFBP-3, IGFBP-5, IGFBP-6, IL-1 alpha, IL-1 beta, IL-2, IL-3, IL-3 Rb, IL-4, IL-5, IL-6, IL-9, IL-10, IL-12 p40/p70, IL-12 p70, IL-13, IL-17, KC, Leptin R, LEPTIN(OB), LIX, L-Selectin, Lymphotactin, MCP-1, MCP5, M-CSF, MIG, MIP-1 alpha, MIP-1 gamma, MIP-2, MIP-3 beta, MIP-3 alpha, PF-4, P-Selectin, RANTES, SCF, SDF-1 alpha, TARC, TCA-3, TECK, TIMP-1, TNF RI, TNF RII, TPO, VCAM-1, VEGF.

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## Troubleshooting Guide

| Problem | Potential Cause | Solution |
|---|---|---|
| Poor Standard Curve | ◦Inaccurate pipetting<br>◦Improper standard dilution | ◦Check pipettes<br>◦Briefly centrifuge the standard protein and dissolve the powder thoroughly by gently mixing |
| Low Signal | ◦Improper preparation of standard and/or biotinylated antibody | ◦Briefly spin down vials before opening |
| | ◦Too brief incubation times | ◦Ensure sufficient incubation times are followed. Standard/sample incubation step may be done overnight at 4ºC with gentle shaking (may increase overall signal, including background) |
| | ◦Inadequate reagent volumes or improper dilution | ◦Check pipettes and ensure correct preparation |
| Large CV | ◦Inaccurate pipetting<br>◦Air bubbles in wells | ◦Check pipettes<br>◦Carefully remove air bubbles |
| High Background | ◦Plate is insufficiently washed | ◦Review protocol for proper wash steps. If using a plate washer, ensure all ports are unobstructed. |
| | ◦Contaminated wash buffer | ◦Make fresh wash buffer |
| Low Sensitivity | ◦Improper storage of the kit | ◦Store the standard at -70ºC after reconstitution, others at 2-8 ºC. Keep substrate solution protected from light |
| | ◦Stop Solution issue | ◦Add stop solution to each well before reading plate |

## Warranty

Products are warranted by Bethyl Laboratories, Inc. to meet stated product specifications and to conform to label descriptions when used, handled and stored according to instructions. Unless otherwise stated, this warranty is limited to six months from date of receipt. Bethyl Laboratories sole liability for the product is limited to replacement of the product or refund of the purchase price. Bethyl Laboratories products are supplied for research applications. They are not intended for medicinal, diagnostic or therapeutic use. The products may not be resold, modified for resale or used to manufacture commercial products without prior written approval from Bethyl Laboratories, Inc. For more information, go to our website: https://www.fortislife.com/sales-terms