**INTERPRETATION OF DATA**

Standard curves must be constructed for each assay.

Typical data from using the human Lymphotoxin-alpha /TNF-beta ELISA is illustrated in the graph below. A standard curve can be plotted using computer generated curve-fitting software or manually using graph paper.

Take the average of replicates for standard, control, and sample dilutions and subtract the average of the zero standard or blank replicates. Plot the standard curve by using curve fitting computer software or by plotting the average optical density (OD) for each standard on the y-axis against the concentration on the x-axis. A curve of best fit can then be drawn through the points on the graph. If desired, plotting of the log of the TNF-beta concentration against the log of the OD can linearize the graph allowing a line of best fit to be drawn, so allowing regression analysis. Multiply values for diluted samples by the dilution factors.

**SPECIFICITY**

The human Lymphotoxin-alpha /TNF-beta ELISA does not cross-react with or exhibit interference from human GM-CSF, TNF-alpha, IL-1b, IL-8 or mouse TNF-alpha. Recombinant human TNF-RI and TNF-RII do not cross-react, but may interfere with the assay when at nanogram per mL levels.

**CALIBRATION**

This Human Lymphotoxin-alpha /TNF-beta ELISA is calibrated against purified human cell expressed TNF-betaB produced by GenWay.

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CONTENTS OF KIT
Coating Antibody (Catalog # E8003.1) - 1 vial containing 720 µg of mouse anti-human TNF-beta, to be reconstituted with 1.0 mL of PBS, which may be stored at 2 – 8 °C for up to 2 months. For longer-term storage, dispense into smaller volumes and store between -20 °C and -70 °C in a manual defrost freezer for up to 6 months, if prior to expiry date. Allow at least 15 minutes at ambient temperature (20-25 °C), with gentle mixing, then dilute 1/200 in PBS for coating of plates.

TNF-beta Standard (Catalog # E8003.2) - 1 vial containing 1000 ng/mL of human cell expressed recombinant human TNF-beta when reconstituted with 0.5 mL of distilled or deionized water with 0.05% NaN3. Following reconstitution, mix gently for 15 minutes or longer, at ambient temperature (20-25 °C), before making dilutions. The reconstituted standard may be used immediately or stored at -70 °C. We recommend generating a ten point standard curve, using 2-fold serial dilutions of TNF-beta, in Assay Buffer (see ADDITIONAL SOLUTIONS REQUIRED), commencing at 2000 pg/mL.

Detection Antibody (Catalog # E8003.3) - 1 vial containing 36 µg of biotinylated goat anti-human TNF-beta, to be reconstituted with 1.0 mL of Assay Buffer (see ADDITIONAL SOLUTIONS REQUIRED), which may be stored at 2 – 8 °C for up to 2 months. For longer-term storage, dispense into smaller volumes and store between -20 °C and -70 °C in a manual defrost freezer for up to 6 months, if prior to expiry date. Allow at least 15 minutes at ambient temperature (20-25 °C), with gentle mixing, then dilute 1/200 in Assay Buffer for use.

Streptavidin-HRP (Catalog # E1999) - 1 vial containing 1.0 mL of streptavidin conjugated to horseradish-peroxidase, which may be stored between 2 – 8 °C for up to 6 months, if prior to expiry date. Dilute 1/200 for use, in Assay Buffer.

ENSURE ALL REAGENTS ARE AT AMBIENT TEMPERATURE (20-25 °C) PRIOR TO USE.

ADDITIONAL SOLUTIONS REQUIRED
Phosphate Buffered Saline (PBS) - 137 mM NaCl, 2.7 mM KCl, 8.1 mM Na2HPO4, 1.5 mM KH2PO4, pH 7.2 - 7.4, 0.2 µm filtered.
Plate Washing Buffer - 0.05% Tween 20® (ICN America) in PBS, pH 7.2 - 7.4.
Assay Buffer - 1% bovine serum albumin (BSA) in PBS, pH 7.2 - 7.4, 0.2 µm filtered.
Substrate Solution - 1:1 (volume) mixture of H2O2 and Tetrathylbenzidine (TMB) (Sigma Cat # T8665).
Stop Solution - 2 N H2SO4 (Wear protective glasses, gloves and clothing when handling).

PRECAUTIONS
All samples and reagents should be brought to ambient temperature (20-25 °C) before use. Thaw all samples and reagents at room temperature.
Dilute standards and samples in culture medium, when assaying tissue culture samples (e.g. RPMI1640/10% fetal calf serum (FCS)) should be used for supernatants from cells grown in this medium).
Use a new disposable reagent reservoir for each step, when using multichannel pipettors.
New disposable pipette tips should be used for each sample or dilution to avoid cross-contamination.
Use new plate sealer for each incubation step.
DO NOT let plates DRY during the assay.
DO NOT USE reagents that are CLOUDY, or BLUE or have of evidence of a CRUST or PRECIPITATE.
Wash plates vigorously for optimal results.
Avoid exposing reagents to temperatures above 37 °C or to bright light during storage or incubations.
Discard unused kit components. Do not mix reagents from different kit lots.
Do not use glass pipettes to measure TMB Substrate Solution. Take care not to contaminate the solution. If the solution is blue before use, DO NOT USE IT.

Wear gloves while performing the assay to avoid exposure to antibiotics, preservatives or contaminated samples and follow proper disposal procedures after use.
Wear protective glasses, gloves and clothing when handling Substrate Solution or Stop Solution (2 N H2SO4).

ELISA PROCEDURE
1. Dilute the Coating Antibody to the working concentration in PBS (no carrier protein included).
   Coat a 96-well microplate (1) with 100 µL per well of the prediluted Coating Antibody, by sealing the plate and incubating for 2 hours at 20-25 °C.
2. After coating, on the next day, aspirate all wells and wash each well with Plate Washing Buffer, three times by filling all wells (370 µL) using a squeeze bottle, multi-channel pipettor, manifold dispenser or automated plate washer. Remove all of the liquid from wells, between each wash, or prior to other steps, by aspirating or by inverting the plate and pressing it onto clean, absorbent paper.
3. Block all wells by adding 300 µL of Assay Buffer to each and incubating for 2 hours at ambient temperature (20-25 °C) or between 36-38 °C for 1 hour.
4. Wash again as in step 2, before addition of samples.
5. Add samples or standards (100 µL/well), prediluted in Assay Buffer or other appropriate diluent, to wells and incubate the plate, after sealing, for 2 hours at ambient temperature (20-25 °C) or between 36-38 °C for 1 hour.
6. Wash again as in step 2, before addition of Detection Antibody.
7. Add Detection Antibody (100 µL), prediluted in Assay Buffer, to all wells and incubate the plate, after sealing, for 2 hours at ambient temperature (20-25 °C) or between 36-38 °C for 1 hour.
8. Wash all wells again as in step 2, before addition of Streptavidin-HRP.
9. Add prediluted Streptavidin-HRP (100 µL) to each well. Cover the plate and incubate for 20 minutes at ambient temperature (20-25 °C). Avoid placing the plate in direct light.
10. Wash all wells again as in step 2, before addition of Substrate Solution.
11. Add Substrate Solution (100 µL) to all wells and incubate the plate after sealing, for 30 minutes at ambient temperature (20-25 °C).
12. Add Stop Solution (50 µL) to all wells, allowing thorough mixing (e.g. by tapping the plate) before reading.
13. Read the absorbance of wells at a wavelength of 450 nm as soon as is practical, using a microplate reader. If possible, subtract readings at 540 nm or 570 nm from the readings at 450 nm, in order to correct for optical imperfections in the plate and improve accuracy.

FURTHER NOTES
1. Adequate and consistent washing is essential for acceptable assay performance (see Assay Procedure).
2. Ensure that new or clean disposable ware is used for each step.
3. Standards and samples should be assayed in duplicate, at the very least.
4. Contaminated reagents or buffers, may compromise the accuracy of assays.
5. Buffers containing proteins should be sterile and stored at 2 - 8 °C or be prepared each day.
6. For serum samples, laboratories should develop a suitable serum diluent, such as PBS/10% fetal calf serum (FCS).
7. Buffers containing serum (e.g. FCS) should not be used for dilution of the standard and test samples must be compatible with the samples being tested. The Assay Buffer listed above in Additional Solutions Required should be adequate for culture supernates. Diluents should be evaluated with samples prior to test assays. For serum samples, laboratories should develop a suitable serum diluent, such as PBS/10% fetal calf serum (FCS).
8. Buffers to be used for reconstitution or dilution of the standard and test samples must be compatible with the samples being tested. The Assay Buffer listed above in Additional Solutions Required should be adequate for culture supernates. Diluents should be evaluated with samples prior to test assays. For serum samples, laboratories should develop a suitable serum diluent, such as PBS/10% fetal calf serum (FCS).
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(1) Costar High Binding ELISA Plate (Cat. # 9018) is recommended, but this kit can be used with anti-mouse immunoglobulin coated plates (e.g. R&D Systems, Cat. # CP001), eliminating the plate coating step.

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