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Introduction

Tumor necrosis factor (TNF, formerly known as TNF-α) is a potent mediator of immune and inflammatory responses. TNF is produced by many activated cell types including monocytes, macrophages, astrocytes, granulocytes, T and B lymphocytes, NK cells, keratinocytes, fibroblasts, and certain tumor cells. TNF exerts many regulatory influences on the activation, growth, and differentiation of leukocytes and other cells. For example, TNF can costimulate the proliferation of activated T and B lymphocytes, upregulate the expressed levels of MHC class I and class II molecules by various cell types, as well as induce the expression of adhesion molecules by endothelial cells. TNF is selectively cytotoxic for some transformed cell lines and can exert cytotoxic effects against certain solid tumors. In vivo, TNF serves as a primary mediator in protective immune responses against microbial and viral pathogens. However, TNF has also been implicated as a central mediator in a number of pathologic responses including septic shock, cachexia, and autoimmune diseases.1-2

Activated cells initially express TNF as transmembrane proteins that associate to form homotrimeric complexes. After proteolytic cleavage, the extracellular region of membrane TNF sheds as a soluble homotrimer. Membrane and soluble TNF homotrimers are biologically active, whereas monomeric TNF is not. The mature mouse TNF monomer contains 156 amino acid residues, has a predicted size of ~17 kDa, and contains one potential N-glycosylation site. TNF exerts its biological activities by binding and signaling through membrane Type I and II TNF Receptors (TNFRI and TNFRII). Interestingly, TNF binds to soluble forms of TNFR (sTNFRI and sTNFRII) which are naturally shed by activated cells and can modulate biological activities of TNF.

The BD OptEIA™ Mouse TNF ELISA Kit is for the quantitative determination of mouse TNF in serum, plasma, and cell culture supernatant.

Principle of the Test

The BD OptEIA test is a solid phase sandwich ELISA (Enzyme-Linked Immunosorbent Assay). It utilizes a monoclonal antibody specific for mouse TNF coated on a 96-well plate. Standards and samples are added to the wells, and any TNF present binds to the immobilized antibody. The wells are washed, and biotinylated polyclonal anti-mouse TNF antibody is added, producing an antibody-antigen-antibody "sandwich." After a second wash, streptavidin-horseradish peroxidase conjugate is added. The wells are again washed and TMB substrate solution is added, which produces a blue color in direct proportion to the amount of TNF present in the initial sample. The Stop Solution changes the color from blue to yellow, and the wells are read at 450 nm.

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Reagents Provided

Antibody Coated Wells: 2 plates of 96 breakable wells (12 strips × 8 wells) coated with anti-mouse TNF monoclonal antibody

Detection Antibody: 30 mL of biotinylated anti-mouse TNF phage Fab antibody with fetal bovine serum (FBS) and with 0.1% ProClin®-150 as a preservative

Standards: 4 vials of lyophilized recombinant mouse TNF

Enzyme Concentrate (250×): 150 μL of 250× concentrated Streptavidin-horseradish peroxidase conjugate with bovine serum albumin (BSA)* and ProClin®-150 as a preservative

Standard/Sample Diluent: 30 mL of animal serum* with 0.09% sodium azide as preservative

Enzyme Diluent: 30 mL of animal serum* base with 0.15% ProClin®-150 as a preservative

ELISA Diluent: 12 mL of a buffered protein base with 0.09% sodium azide as preservative

Wash Concentrate (20×): 100 mL of 20× concentrated detergent solution with ProClin®-150 as a preservative

TMB One-Step Substrate Reagent: 30 mL of 3,3',5,5'-tetramethylbenzidine (TMB) in buffered solution

Stop Solution: 13 mL of 1 M phosphoric acid

Plate Sealers: 4 sheets with adhesive backing

*Source of all serum proteins is from USDA inspected abattoirs located in the United States
Materials Required but not Provided

- Microplate reader capable of measuring absorbance at 450 nm
- Precision pipettes to deliver 50 μL and 100 μL volumes
- Adjustable 1 mL, 5 mL, 10 mL, 25 mL pipettes for reagent preparation
- Deionized or distilled water
- Wash bottle or automated microplate washer
- Log-log graph paper or computer and software for ELISA data analysis
- Tubes to prepare standard dilutions
- Laboratory timer
- Absorbent paper

Storage Information

1. Store the unopened kit at 2 - 8°C. Do not use the kit after the expiration date.
2. Before use, bring all reagents to room temperature (18 - 25°C). Immediately after use, return to proper storage conditions.
3. Lyophilized standards are stable until the kit expiration date. After reconstitution, use freshly reconstituted standard within 12 hours (stored at 2 - 8°C).

Warnings and Precautions

1. Reagents that contain preservatives may be toxic if ingested, inhaled, or brought in contact with skin.
2. Avoid contact of skin, eyes, or clothing with Stop Solution or Substrate Reagents.
3. Handle all serum and plasma specimens in accordance with CLSI guidelines for preventing transmission of blood-borne infections.
4. Standard/Sample Diluent and ELISA Diluent contain less than 0.1% sodium azide. Sodium azide yields highly toxic hydrazoic acid under acidic conditions. Dilute azide compounds in running water before discarding to avoid accumulation of potentially explosive deposits in plumbing.

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5. **Warning**

Wash Concentrate (20X) (component 51-9003738) contains 0.002% (w/w), Mouse TNF Lyophilized Standard (component 51-26676E) contains 0.03% (w/w), Enzyme Diluent (component 51-2718KD) contains 0.003% (w/w) and Detection Antibody Biotin Anti-Mouse TNF (component 51-9006279) contains 0.003% (w/w) of a CMIT/MIT mixture (3:1), which is a mixture of: 5-chloro-2-methyl-4-isothiazolin-3-one [EC No 247-500-7] and 2-methyl-4-isothiazolin-3-one [EC No 220-239-6] (3:1).

**Hazard statements**

*May cause an allergic skin reaction.*

**Precautionary statements**

*Wear protective gloves / eye protection.*

*Wear protective clothing.*

*Avoid breathing mist/vapours/spray.*

*If skin irritation or rash occurs: Get medical advice/attention.*

*IF ON SKIN: Wash with plenty of water.*

*Dispose of contents/container in accordance with local/regional/national/international regulations.*

6. **Danger**

Stop Solution (component 51-2608KC) contains 15.23% phosphoric acid (w/w).

**Hazard statements**

*Causes severe skin burns and eye damage.*

**Precautionary statements**

*Wear protective gloves / eye protection.*

*Wear protective clothing.*

*IF ON SKIN (or hair): Remove/Take off immediately all contaminated clothing. Rinse skin with water/shower.*

*IF IN EYES: Rinse cautiously with water for several minutes. Remove contact lenses, if present and easy to do.*

*Continue rinsing.*

*IF INHALED: Remove victim to fresh air and keep at rest in a position comfortable for breathing.*

*Dispose of contents/container in accordance with local/regional/national/international regulations.*

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Specimen Collection and Handling

Specimens should be clear, non-hemolyzed, and non-lipemic. Samples with expected values higher than the top standard, 2000 pg/mL, should be diluted with Standard/Sample Diluent prior to running the assay.

Cell culture supernatants: Remove any particulate material by centrifugation and assay immediately or store samples at ≤ -20°C. Avoid repeated freeze-thaw cycles.

Serum: Use a serum tube (eg, BD Vacutainer® Cat. No. 366430) and allow samples to clot for 30 minutes, then centrifuge for 10 minutes at 1000 × g. Remove serum and assay immediately or store samples at ≤ -20°C. Avoid repeated freeze-thaw cycles.

Plasma: Collect plasma using citrate, EDTA, or heparin as anticoagulant. Centrifuge for 10 minutes at 1000 × g within 30 minutes of collection. Assay immediately or store samples at ≤ -20°C. Avoid repeated freeze-thaw cycles.

Reagent Preparation

1. Bring all reagents to room temperature (18 - 25°C) before use.
2. Standards
   a. Reconstitute 1 vial lyophilized Standard with the required volume (noted on vial label) of Standard/Sample Diluent to prepare a 2000 pg/mL stock standard. Allow the standard to equilibrate for at least 15 minutes before making dilutions. Vortex to mix.
   b. Add 300 μL Standard/Sample Diluent to 6 tubes. Label the tubes as 1000 pg/mL, 500 pg/mL, 250 pg/mL, 125 pg/mL, 62.5 pg/mL, and 31.3 pg/mL.
   c. Perform serial dilutions by adding 300 μL of each standard to the next tube and vortexing between each transfer. The undiluted standard serves as the high standard (2000 pg/mL). The Standard/Sample Diluent serves as the zero standard (0 pg/mL).

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3. Wash Buffer

Note: If the Wash Concentrate contains visible crystals, warm to room temperature and mix gently until dissolved. Dilute the required quantity of 20× Wash Concentrate with deionized or distilled water and mix. (To prepare 2,000 mL, add 100 mL Wash Concentrate to 1,900 mL water. At least 500 mL solution should be prepared for a full 96-well plate).

4. TMB One-Step Substrate Reagent

No more than 15 minutes prior to use, add required volume of TMB One-Step Substrate Reagent to a clean tube or reservoir. To prevent contamination, pipette out from the tube/reservoir instead of directly from bottle. Avoid prolonged exposure to light or contact with metal, air, or extreme temperature as color may develop.

Assay Procedure

1. Bring all reagents and samples to room temperature (18 - 25°C) prior to use. It is recommended that all standards and samples be run in duplicate.

2. Remove the required quantity of test strips/wells and place in well holder.

Note: Wells are provided in breakable 8-well strips. Strips may be “broken” into individual wells, replaced in a well holder, and assayed. Return any unused wells to sealed pouch for 2 - 8°C storage.

3. Pipette 50 μL of ELISA Diluent into each well.

4. Pipette 50 μL of each standard (see Reagent Preparation, step 2) and sample into appropriate wells. Cover wells with Plate Sealer and incubate for 2 hours at room temperature.

5. Decant or aspirate contents of wells. Wash wells by filling with at least 300 μL/well prepared Wash Buffer (see Reagent Preparation, step 3) followed by decanting/aspirating. Repeat the wash 4 times for a total of 5 washes. After the last wash, blot the plate on absorbent paper to remove any residual buffer. Complete removal of liquid is required for proper performance.

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6. Add 100 \( \mu L \) of Detection Antibody to each well. Cover wells with Plate Sealer and incubate for 1 hour at room temperature.

   
b. Pipette the required volume of Enzyme Diluent into a clean tube or flask. Add in the required quantity of Enzyme Concentrate (250×) and vortex or mix well. For a full 96-well plate, add 48 \( \mu L \) of Enzyme Concentrate into 12 mL of Enzyme Diluent.

8. Wash wells as in Step 5.

9. Add 100 \( \mu L \) of Enzyme Working Reagent (see step 7 above) to each well. Cover wells with Plate Sealer and incubate for 30 minutes at room temperature.

10. Wash wells as in Step 5, but a total of 7 times.
    
    **Note:** In this final wash step, soak wells in wash buffer for 30 seconds to 1 minute for each wash. Thorough washing at this step is very important.

11. Add 100 \( \mu L \) of TMB One-Step Substrate Reagent to each well. Incubate plate (without Plate Sealer) for 30 minutes at room temperature in the dark.

12. Add 50 \( \mu L \) of Stop Solution to each well.

13. Read absorbance at 450 nm within 30 minutes of stopping the reaction. If wavelength correction is available, subtract the optical density readings at 570 nm from readings at 450 nm.

A standard curve is required in each assay run.
Assay Procedure Summary

1. Add 50 μL ELISA Diluent to each well.
2. Add 100 μL standard or sample to each well.
   Incubate 2 hours at room temperature.
3. Aspirate and wash 5 times.
4. Add 100 μL Detection Antibody to each well.
   Incubate 1 hour at room temperature.
5. Aspirate and wash 5 times.
6. Add 100 μL Enzyme Working Reagent to each well.
   Incubate 30 minutes at room temperature.
7. Aspirate and wash/soak 7 times.
8. Add 100 μL TMB One-Step Substrate Reagent to each well.
   Incubate 30 minutes at room temperature.
9. Add 50 μL Stop Solution to each well.
   Read at 450 nm within 30 minutes.
   λ correction 570 nm.
Calculation of Results

Calculate the mean absorbance for each set of duplicate standards, controls, and samples. Subtract the mean zero standard absorbance (ie, plate background) from each.

Plot the standard curve on log-log graph paper, with TNF concentration on the x-axis and absorbance on the y-axis. Draw the best fit straight line through the standard points.

To determine the TNF concentration of the unknowns, find the unknowns’ mean absorbance value on the y-axis and draw a horizontal line to the standard curve. At the point of intersection, draw a vertical line to the x-axis and read the TNF concentration. If samples were diluted, multiply the interpolated TNF concentration by the dilution factor.

Computer-based curve-fitting statistical software may also be employed.

Typical Data

This standard curve is for demonstration only. A standard curve must be run with each assay.

<table>
<thead>
<tr>
<th>Concentration (pg/mL)</th>
<th>OD1</th>
<th>OD2</th>
<th>Mean</th>
<th>Zero Standard Subtracted</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0.027</td>
<td>0.027</td>
<td>0.027</td>
<td>0.000</td>
</tr>
<tr>
<td>31.3</td>
<td>0.103</td>
<td>0.104</td>
<td>0.104</td>
<td>0.077</td>
</tr>
<tr>
<td>62.5</td>
<td>0.171</td>
<td>0.162</td>
<td>0.167</td>
<td>0.140</td>
</tr>
<tr>
<td>125</td>
<td>0.317</td>
<td>0.308</td>
<td>0.312</td>
<td>0.286</td>
</tr>
<tr>
<td>250</td>
<td>0.596</td>
<td>0.619</td>
<td>0.607</td>
<td>0.580</td>
</tr>
<tr>
<td>500</td>
<td>1.042</td>
<td>1.089</td>
<td>1.066</td>
<td>1.039</td>
</tr>
<tr>
<td>1000</td>
<td>1.932</td>
<td>1.934</td>
<td>1.933</td>
<td>1.906</td>
</tr>
</tbody>
</table>
Limitations of the Procedure

1. This kit is intended for use as an integral unit. Do not mix reagents from different kit lots. Reagents from other manufacturers and other available clones should not be used in this kit.

2. Interference by drug metabolites, soluble receptors, or other binding proteins in specimens has not been thoroughly investigated. The possibility of interference cannot be excluded.

Performance Characteristics

Limit of Detection

The minimum detectable dose of TNF was determined to be 1.07 pg/mL. This is defined as two standard deviations above the mean optical density of 20 replicates of the zero standard.

Recovery

Three different amounts of TNF were spiked into pooled mouse serum and cell culture media. Results are compared with the same amounts of TNF spiked in Standard/Sample Diluent, as follows:

<table>
<thead>
<tr>
<th>Spike Concentration (pg/mL)</th>
<th>Average % Recovery</th>
<th>Range</th>
</tr>
</thead>
<tbody>
<tr>
<td>Serum (n = 5)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1000</td>
<td>101</td>
<td>85 - 109</td>
</tr>
<tr>
<td>500</td>
<td>106</td>
<td>95 - 112</td>
</tr>
<tr>
<td>250</td>
<td>111</td>
<td>96 - 117</td>
</tr>
<tr>
<td>Cell culture media (n = 3)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>500</td>
<td>110</td>
<td>106 - 116</td>
</tr>
<tr>
<td>250</td>
<td>107</td>
<td>104 - 108</td>
</tr>
<tr>
<td>125</td>
<td>97</td>
<td>93 - 104</td>
</tr>
</tbody>
</table>

Linearity

Five pooled mouse serum samples and three cell culture media samples were spiked with recombinant TNF, serially diluted with Standard/Sample-Diluent, and run using the BD OptEIA Mouse TNF ELISA kit. Results are as follows:

<table>
<thead>
<tr>
<th>Dilution</th>
<th>Pooled Serum (n = 5)</th>
<th>Cell culture media (n = 3)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1:2</td>
<td>Average % of Expected Range</td>
<td>107 103 - 109</td>
</tr>
<tr>
<td></td>
<td></td>
<td>93 - 114</td>
</tr>
<tr>
<td></td>
<td></td>
<td>111 106 - 116</td>
</tr>
<tr>
<td>1:4</td>
<td>Average % of Expected Range</td>
<td>109 109</td>
</tr>
<tr>
<td></td>
<td></td>
<td>94 - 116</td>
</tr>
<tr>
<td></td>
<td></td>
<td>109 104 - 114</td>
</tr>
<tr>
<td>1:8</td>
<td>Average % of Expected Range</td>
<td>114 99</td>
</tr>
<tr>
<td></td>
<td></td>
<td>100 - 121</td>
</tr>
<tr>
<td></td>
<td></td>
<td>99 88 - 107</td>
</tr>
<tr>
<td>1:16</td>
<td>Average % of Expected Range</td>
<td>117 103</td>
</tr>
<tr>
<td></td>
<td></td>
<td>100 - 124</td>
</tr>
<tr>
<td></td>
<td></td>
<td>97 - 110</td>
</tr>
</tbody>
</table>

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Specificity

Cross Reactivity: The following factors were tested using the BD OptEIA assay at \( \geq 10 \) ng/mL, and no cross-reactivity (value \( \geq 15 \) pg/mL) was identified.

Recombinant Human

TNF

Recombinant Mouse

IL-1\( \alpha \), IL-1\( \beta \), IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-9, IL-10, IL-12 (p70), IL-13, IL-15, IL-17, IL-18, GM-CSF, IFN-\( \gamma \), LT-\( \alpha \), MCP-1, M-CSF, MIG, MIP-1\( \alpha \), MCIP-1\( \beta \), sTNFRI, sTNFRII, RANTES

Recombinant Rat

IL-1\( \alpha \), IL-2, IL-4, IL-6, IL-10, IL-18, GM-CSF, IFN-\( \gamma \)

Precision

Intra-assay

Twenty-four replicates each of three different levels of TNF were tested in one plate. The following results were observed:

<table>
<thead>
<tr>
<th>Number of Replicates</th>
<th>24</th>
<th>24</th>
<th>24</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean Concentration</td>
<td>585.5 pg/mL</td>
<td>287.4 pg/mL</td>
<td>135.8 pg/mL</td>
</tr>
<tr>
<td>SD</td>
<td>34.2</td>
<td>13.9</td>
<td>14.8</td>
</tr>
<tr>
<td>%CV</td>
<td>5.8</td>
<td>4.8</td>
<td>10.9</td>
</tr>
</tbody>
</table>

Inter-assay

Three different levels of TNF were tested in four different plates. The following results were observed:

<table>
<thead>
<tr>
<th>Number of Replicates</th>
<th>32</th>
<th>32</th>
<th>32</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean Concentration</td>
<td>555.3 pg/mL</td>
<td>277.2 pg/mL</td>
<td>136.1 pg/mL</td>
</tr>
<tr>
<td>SD</td>
<td>46.7</td>
<td>24.2</td>
<td>16.8</td>
</tr>
<tr>
<td>%CV</td>
<td>8.4</td>
<td>8.7</td>
<td>12.4</td>
</tr>
</tbody>
</table>

Standardization

The immunoassay was calibrated against recombinant mouse TNF.

During the development of this product, the NIBSC/WHO mouse TNF reference Standard 88/532 (recombinant mouse TNF) was evaluated in this kit. The conversion factor for NIBSC material is as follows:

\[
1 \text{ mg NIBSC (88/532) TNF} = 0.3 \text{ BD OptEIA recombinant mouse TNF}
\]
Experimental Results

Serum

Seventeen apparently healthy normal mouse serum samples were tested in this assay. Fourteen samples measured less than 31.3 pg/mL (lowest standard level). Three samples measured above the lowest standard at 36.5, 37.3, and 73.5 pg/mL.

Cell Culture Supernatants

Mouse splenocytes or peritoneal exudate cells (PECs) from apparently healthy, normal mice were cultured in RPMI 1640 complete medium with 7.5% FBS at 1 x 10^6 cells/mL, and stimulated with Con A (5 μg/mL) for 24 hours (1), or IFN-γ (10 ng/mL)/LPS (1 μg/mL) for 7 days (2), or PMA/ionomycin for 24 hours (3) or 48 hours (4). PECs were differentiated into MiCK-2 cells (5). Culture supernatants were collected and quantified for TNF using the BD OptEIA Mouse TNF ELISA Kit. The results are as follows:

<table>
<thead>
<tr>
<th>Mouse No.</th>
<th>TNF (pg/mL)</th>
<th>Details</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>182</td>
<td>Balb/c splenocytes, 24 hr Con A (5 μg/mL) stimulation</td>
</tr>
<tr>
<td>2</td>
<td>2,247</td>
<td>PEC, 7 day Ms IFN (10 ng/mL) + LPS (1 μg/mL) stimulation</td>
</tr>
<tr>
<td>3</td>
<td>1,215</td>
<td>C57BL/6 splenocytes, 24 hr PMA/ionomycin stimulation</td>
</tr>
<tr>
<td>4</td>
<td>2,251</td>
<td>C57BL/6 splenocytes, 48 hr PMA/ionomycin stimulation</td>
</tr>
<tr>
<td>5</td>
<td>21,458</td>
<td>MiCK-2 cells, (6 hr PMA (5 ng/mL) + Ionomycin (500 μg/mL) stimulation</td>
</tr>
</tbody>
</table>

Troubleshooting

<table>
<thead>
<tr>
<th>Problem</th>
<th>Possible Source</th>
<th>Corrective Action</th>
</tr>
</thead>
<tbody>
<tr>
<td>Poor Precision</td>
<td>• Inadequate washing / aspiration of wells&lt;br&gt;• Inadequate mixing of reagents&lt;br&gt;• Imprecise / inaccurate pipetting&lt;br&gt;• Imprecise sealing of plate</td>
<td>• Check function of washing system&lt;br&gt;Ensure adequate mixing&lt;br&gt;Check / calibrate pipettes&lt;br&gt;Ensure complete sealing of plate</td>
</tr>
<tr>
<td>Poor Standard Curve</td>
<td>• Improper standard handling / dilution&lt;br&gt;• Incomplete washing / aspiration of wells&lt;br&gt;• Imprecise / inaccurate pipetting</td>
<td>• Ensure correct preparation of standards&lt;br&gt;Check function of washing system&lt;br&gt;Check / calibrate pipettes</td>
</tr>
<tr>
<td>Low Signal</td>
<td>• Inadequate reagent volumes added to wells&lt;br&gt;• Incorrect incubation times / temperature&lt;br&gt;• Overly high wash / aspiration pressure from automated plate-washer.</td>
<td>• Check / calibrate pipettes&lt;br&gt;Ensure sufficient incubation times / reagents warmed to room temperature&lt;br&gt;Utilize manual washing</td>
</tr>
</tbody>
</table>
References

