Calculation of Results
Calculate the mean absorbance for each set of duplicate standards, controls and samples. Subtract the mean zero standard absorbance 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 curve through the standard points.

To determine the TNF concentration of the unknowns, find the unknown's mean absorbance value on the x-axis and draw a vertical line to the y-axis and read the TNF concentration. If samples were diluted, multiply the TNF concentration by the dilution factor.

Computer data reduction may also be employed, utilizing log-log regression analysis.

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

Mouse TNF (pg/mL)

<table>
<thead>
<tr>
<th>Mouse TNF (pg/mL)</th>
<th>0</th>
<th>0.1</th>
<th>0.5</th>
<th>1</th>
<th>2</th>
<th>4</th>
<th>10</th>
<th>50</th>
<th>100</th>
<th>500</th>
<th>1000</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.0</td>
<td>1</td>
<td>2</td>
<td>3</td>
<td>4</td>
<td>5</td>
<td>6</td>
<td>7</td>
<td>8</td>
<td>9</td>
<td>10</td>
<td>15</td>
</tr>
</tbody>
</table>

Standardization
This immunoassay is calibrated against purified Baculovirus-expressed recombinant mouse TNF.

The NIBSC/WHO Reference Standard 88/532 (recombinant mouse TNF) was evaluated in this set. The conversion factor for NIBSC material is as follows:

1 µg NIBSC 88/532 mouse TNF = 0.61 µg BD OptEIA™ mouse TNF

Assay Optimization
1. BD OptEIA™ Sets allow flexible assay design to fit individual laboratory needs. To design an immunoassay with different sensitivity and dynamic range, the following parameters can be varied: Capture, Detection Antibody titers, Incubation time, Incubation temperature, Assay Diluent formulation, Buffer pH, ionic strength, protein concentration, Type of substrate, Washing technique (i.e., number of wash repetitions and soak times)

2. “Typical Standard Curve” and 20-plate yield were obtained in the BD Biosciences Pharmingen laboratory, using the recommended procedure and manual plate washing.

Troubleshooting

Poor Precision
Possible Source
• Inadequate washing/aspiration of wells
• Inadequate mixing of reagents
• Imprecise/inaccurate pipetting
• Incomplete sealing of plate

Corrective Action
• Check function of washing system
• Ensure adequate mixing
• Check/pipette calibration
• Ensure complete seal on plate

Poor Standard Curve
Possible Source
• Improper standard handling/dilution
• Incomplete washing/aspiration of wells
• Imprecise/inaccurate pipetting
• Improper buffer/diluent used

Corrective Action
• Ensure correct preparation, storage of standards
• Check function of washing system
• Check/calibrate pipettes
• Check buffer/diluent preparation, pH

Low Absorbances
Possible Source
• Inadequate reagent volumes added to wells
• Incorrect incubation times/temperature
• Incorrect antibody titration
• Improper buffer/diluent used
• Overly high wash/ aspiration pressure from automated plate-washer

Corrective Action
• Check/calibrate pipettes
• Ensure sufficient incubation times/ reagents warmed to RT
• Check/validate Capture Ab and Working Detector preparation
• Check buffer/diluent preparation, pH
• Utilize manual washing

Specificity

Cross Reactivity:

The following factors were tested in the BD OptEIA™ assay at ≥ 10 ng/mL and no cross-reactivity (value ≥ 4 pg/mL) was identified.

Recombinant Human
IL-1α, IL-1β, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-11, IL-12 (p40), IL-12 (p70), IL-13, IL-15, G-CSF, GM-CSF, IFN-γ, CD23, Lymphotactin, MIP-1α, MIP-1β, MCP-1, MCP-2, NT-3, PDGF-AA, SCF, TNF, LT-α (TNF-β), VEGF

Recombinant Mouse
IL-1β, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-9, IL-10, IL-12 (p70), IL-15, IFN-γ, GM-CSF, MCP-1, TCA3

Recombinant Rat
IL-2, IL-4, IL-6, IL-10, GM-CSF, IFN-γ, TNF

Other:

Viral IL-10 (1 ng/mL), Rabbit TNF

Limitations of the Procedure

• Samples that generate absorbance values higher than the standard curve should be diluted with Standard Diluent and re-assayed.
• Interference by drug metabolites, soluble receptors, or other binding proteins in specimens has not been thoroughly investigated. The possibility of interference cannot be excluded.
• BD OptEIA™ Sets are intended for use as an integral unit. Do not mix reagents from different Set batches. Reagents from other manufacturers are not recommended for use in this Set.

References

Recommended buffers, solutions

Note: Do not use sodium azide in these preparations. Sodium azide inactivates the horseradish peroxidase enzyme.

The BD OptEIA™ Reagent Set A (Cat. No. 550536) containing Coating Buffer, Assay Diluent, Substrate Reagents A and B, Stop Solution and 20X Wash Buffer Concentrate is recommended.

1. **Coating Buffer**: 0.2 M Sodium Phosphate, pH 6.5
   11.8 g Na₂HPO₄, 16.1 g NaH₂PO₄, q.s. to 1.0 L; pH to 6.5.

2. **Assay Diluent**: PBS* with 10% FBS*, pH 7.0. The BD Pharmingen™ Assay Diluent (Cat. No. S55213) is recommended.

3. **Wash Buffer**: PBS* with 0.05% Tween-20. Freshly prepare or use within 3 days of preparation, stored at 2-8°C.

4. **Substrate Solution**: Tetramethylbenzidine (TMB) and Hydrogen Peroxide. The BD Pharmingen™ TMB Substrate Reagent Set (Cat. No. S55214) is recommended.

5. **Stop Solution**: 1 M H₃PO₄ or 2 N H₂SO₄

Additional Materials Required

1. 96-well BD Falcon™ ELISA plates (Cat. No. 353279) are recommended.

2. Microplate reader capable of measuring absorbance at 450 nm

3. Precision pipettes

4. Graduated cylinder, one liter

5. Deionized or distilled water

6. Wash bottle or automated washer

7. Log-log graph paper or automated data reduction

8. Tubes to prepare standard dilutions

9. Laboratory timer

10. Plate sealers or parafilm

Storage Information

1. Store unopened reagents at 2-8°C. Do not use reagents after expiration date, or if turbidity is evident.

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 expiration date. See below for reconstituted standard storage information.

Specimen Collection and Handling

Specimens should be clear, non-hemolyzed and non-lipemic.

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 separator tube and allow samples to clot for 30 minutes, then centrifuge for 10 minutes at 1000 x 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 x g within 30 minutes of collection. Assay immediately or store samples at ≤ -20°C. Avoid repeated freeze-thaw cycles.

Serum and plasma specimens should be stored in the United States.

Recommended Assay Procedure

1. **Reconstitution**: After warming lyophilized standard to room temperature, carefully open vial to avoid loss of material. Reconstitute lyophilized standard with 1.0 mL of deionized water to yield a stock standard. Allow the standard to equilibrate for at least 15 minutes before making dilutions. Vortex gently to mix.

2. **Storage/ handling of reconstituted standard**: After reconstitution, immediately aliquot standard stock in polypropylene vials at 50 µL per vial and freeze at -80°C for up to 6 months. If necessary, store at 2-8°C for up to 8 hours prior to aliquoting/freezing. Do not leave reconstituted standard at room temperature.

3. **Standards Preparation for Assay**:
   a. Prepare a 1000 pg/mL standard from the stock standard. Vortex to mix. (See dilution instructions on Instruction/Analysis Certificate.)
   b. Add 300 µL Assay Diluent to 6 tubes. Label as 500 pg/mL, 250 pg/mL, 125 pg/mL, 62.5 pg/mL, 31.3 pg/mL, and 15.6 pg/mL.
   c. Perform serial dilutions by adding 300 µL of each standard to the next tube and vortexing between each transfer. Assay Diluent serves as the zero standard (0 pg/mL).

4. **Washing**: Aspirate wells and wash 3 times with ≥ 300 µL/well Wash Buffer. After last wash, invert plate and blot on absorbent paper to remove any residual buffer.

5. **Block plates** with ≥ 200 µL/well Assay Diluent. Incubate for 1 hour.

6. **Aspirate/ wash** as in step 2.

7. **Prepare standard and sample dilutions in Assay Diluent**.

8. **Aspirate**/wash as in step 2, but with 5 total washes.

9. **Add 100 µL Working Detector (Detection Antibody + SAv-HRP reagent) to each well**. Seal plate and incubate for 1 hour. Reagents which contain preservatives may be toxic if ingested, inhaled, or in contact with skin.

10. **Aspirate**/wash as in step 2, but with 7 total washes.

11. **Add 100 µL of Substrate Solution to each well**. Incubate plate (with-out plate sealer) for 30 minutes at room temperature in the dark. If wavelength correction is available, subtract absorbance at 570 nm from absorbance 450 nm.

Assay Procedure Summary

1. **Add 100 µL diluted Capture Ab to each well**. Incubate overnight at 4°C.

2. **Aspirate and wash** 3 times.

3. **Block plates**: 200 µL Assay Diluent to each well. Incubate 1 hr RT

4. **Aspirate and wash** 3 times.

5. **Add 100 µL standard or sample to each well**. Incubate 2 hr RT.

6. **Aspirate and wash** 5 times.

7. **Add 100 µL Working Detector (Detection Ab + SAv-HRP) to each well**. Incubate 1 hr RT

8. **Aspirate and wash** 7 times

9. **Add 100 µL Substrate Solution to each well**. Incubate 30 min RT in dark

10. **Add 50 µL Stop Solution to each well**. Read at 450 nm within 30 minutes of stopping reaction.

### Recommended assay procedures

- **Capture Antibody**: Diluted in Coating Buffer. For recommended antibody coating dilution, see lot-specific Instruction/Analysis Certificate. Seal plate and incubate overnight at 4°C.

- **Wash**: Aspirate and wash 3 times with ≥ 300 µL/well Wash Buffer. After last wash, invert plate and blot on absorbent paper to remove any residual buffer.

- **Block**: Plates with ≥ 200 µL/well Assay Diluent. Incubate for 1 hour.

- **Aspirate/ wash** as in step 2.

- **Prepare standard and sample dilutions in Assay Diluent**. See “Standards Preparation and Handling.”

- **Add 100 µL Working Detector (Detection Antibody + SAv-HRP reagent) to each well**. Seal plate and incubate for 1 hour.

- **Aspirate/ wash** as in step 2, but with 7 total washes.

- **Add 100 µL of Substrate Solution to each well**. Incubate plate (without plate sealer) for 30 minutes at room temperature in the dark.

- **Add 50 µL of Stop Solution to each well**.

- **Read absorbance at 450 nm within 30 minutes of stopping reaction**.

- **If wavelength correction is available, subtract absorbance at 570 nm from absorbance 450 nm.**

### Standards Preparation and Handling

1. **Reconstitution**: After warming lyophilized standard to room temperature, carefully open vial to avoid loss of material. Reconstitute lyophilized standard with 1.0 mL of deionized water to yield a stock standard. Allow the standard to equilibrate for at least 15 minutes before making dilutions. Vortex gently to mix.

2. **Storage/ handling of reconstituted standard**: After reconstitution, immediately aliquot standard stock in polypropylene vials at 50 µL per vial and freeze at -80°C for up to 6 months. If necessary, store at 2-8°C for up to 8 hours prior to aliquoting/freezing. Do not leave reconstituted standard at room temperature.

### Working Detector Preparation

**Note**: One-step incubation of Biotin/Streptavidin reagents.

<table>
<thead>
<tr>
<th>Standards Preparation for Assay</th>
<th>Coating Buffer</th>
<th>Assay Diluent</th>
<th>Substrate Reagents A and B</th>
<th>Stop Solution</th>
<th>20X Wash Buffer Concentrate</th>
</tr>
</thead>
</table>

### Working Detector Preparation

1. **Add 100 µL diluted Capture Ab to each well**. Incubate overnight at 4°C.

2. **Aspirate and wash** 3 times.

3. **Block plates**: 200 µL Assay Diluent to each well. Incubate 1 hr RT

4. **Aspirate and wash** 3 times.

5. **Add 100 µL standard or sample to each well**. Incubate 2 hr RT.

6. **Aspirate and wash** 5 times.

7. **Add 100 µL Working Detector (Detection Ab + SAv-HRP) to each well**. Incubate 1 hr RT

8. **Aspirate and wash** 7 times

9. **Add 100 µL Substrate Solution to each well**. Incubate 30 min RT in dark

10. **Add 50 µL Stop Solution to each well**. Read at 450 nm within 30 min with λ correction 570 nm.