**CALCULATION OF RESULTS**

Average the duplicate readings for each standard, control, and sample and subtract the average zero standard optical density (O.D.). Create a standard curve by reducing the data using computer software capable of generating a four parameter logistic (4-PL) curve-fit. As an alternative, construct a standard curve by plotting the mean absorbance for each standard on the y-axis against the concentration on the x-axis and draw a best fit curve through the points on the graph. The data may be linearized by plotting the log of the porcine TNF-α concentrations versus the log of the O.D. and the best fit line can be determined by regression analysis. This procedure will produce an adequate but less precise fit of the data. If samples have been diluted, the concentration read from the standard curve must be multiplied by the dilution factor.

**SPECIFICITY**

The following factors prepared at 50 ng/mL were assayed and exhibited no cross-reactivity or interference.

<table>
<thead>
<tr>
<th>Recombinant human:</th>
<th>Recombinant mouse:</th>
<th>Other recombinants:</th>
</tr>
</thead>
<tbody>
<tr>
<td>TNF-β</td>
<td>TNF RI</td>
<td>rat TNF-α</td>
</tr>
<tr>
<td>TNF RI</td>
<td>TNF RI</td>
<td>feline TNF-α</td>
</tr>
<tr>
<td>TNF RII</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

The factors listed below were assayed and exhibited the following cross-reactivities:

<table>
<thead>
<tr>
<th>Recombinant Factor</th>
<th>Concentration Tested (pg/mL)</th>
<th>Observed Value (pg/mL)</th>
<th>% Cross-reactivity</th>
</tr>
</thead>
<tbody>
<tr>
<td>bovine TNF-α</td>
<td>50,000</td>
<td>387</td>
<td>0.8</td>
</tr>
<tr>
<td>canine TNF-α</td>
<td>1250</td>
<td>116</td>
<td>9.3</td>
</tr>
<tr>
<td>equine-TNF-α</td>
<td>2500</td>
<td>298</td>
<td>11.9</td>
</tr>
<tr>
<td>human TNF-α</td>
<td>6250</td>
<td>396</td>
<td>6.3</td>
</tr>
<tr>
<td>Rhesus monocat TNF-α</td>
<td>312</td>
<td>176</td>
<td>56.5</td>
</tr>
</tbody>
</table>

**TECHNICAL HINTS & LIMITATIONS**

- We recommend the use of R&D Systems’ Reagent Diluent Concentrate 2 (Catalog # DY995) to prepare Block Buffer for use in this assay.
- The use of high quality Bovine Serum Albumin (BSA) for the Reagent Diluent and Block Buffer is crucial for the optimum performance of the DuoSet ELISA Development kit. Impurities such as proteases, binding proteins, soluble receptors or other interfering substances can be found to varying degrees in virtually all BSA preparations and can inhibit or interfere with the detection of certain analytes. If the standard curve appears suppressed, consider evaluating a different preparation of BSA.
- It is suggested to start Reagent Diluent optimization for serum and plasma samples by using PBS supplemented with 10-50% animal serum. Do not use buffers with animal serum to reconstitute or dilute the Detection Antibody or Streptavidin-HRP.
- It is important that the Reagent Diluent selected for reconstitution and dilution of the standard reflects the environment of the samples being measured.
- Avoid microbial contamination of reagents and buffers.
- A thorough and consistent wash technique is essential for proper assay performance. Wash Buffer should be dispensed forcefully and removed completely from the wells by aspiration or decanting. Remove any remaining Wash Buffer by inverting the plate and blotting it against clean paper towels.
- Individual results may vary due to differences in technique, plasticware and water sources.
- It is recommended that all standards and samples be assayed in duplicate.
- The use of PBS from tablets may interfere in this assay.

**TYPICAL DATA**

This standard curve is only for demonstration purposes. A standard curve should be generated for each set of samples assayed.

![Typical Data Graph](Image)

**TROUBLESHOOTING**

**Poor Standard Curve**

- Impure BSA used for Reagent Diluent and Block Buffer preparation.
- Improper reconstitution and/or storage of standard.
- Improper dilution of highest standard and standard curve.
- Incomplete washing and/or aspiration of wells.
- Unequal mixing of reagents.

**Poor Precision**

- Unequal volumes added to wells/pipetting error.
- Incomplete washing and/or aspiration of wells.
- Unequal mixing of reagents.

**Low or No Color Development**

- Inadequate volume of substrate added to wells.
- Incorrect incubation times or temperatures.
- Impure BSA used for Reagent Diluent and Block Buffer preparation.

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E-MAIL: info@RnDSystemsChina.com.cn

www.RnDSystems.com
OTHER MATERIALS & SOLUTIONS REQUIRED

DuoSet Ancillary Reagent Kit 2 (5 plates):
(R&D Systems, Catalog # DY008) containing 96 well microplates, plate sealers, substrate solution, stop solution, plate coating buffer (PBS), wash buffer, and Reagent Diluent Concentrate 2.

The components listed above may be purchased separately:
96 well microplates: (R&D Systems, Catalog # DY990).
Plate Sealers: (R&D Systems, Catalog # DY992).
PBS: 137 mM NaCl, 2.7 mM KCl, 8.1 mM Na₃HPO₄, 1.5 mM KH₂PO₄, pH 7.2-7.4, 0.2 μm filtered (R&D Systems, Catalog # DY006).
Wash Buffer: 0.05% Tween® 20 in PBS, pH 7.2-7.4 (R&D Systems, Catalog # WA126).
Reagent Diluent: 0.1% BSA, 0.05% Tween 20 in Tris-buffered Saline (20 mM Trizma base, 150 mM NaCl) pH 7.2-7.4, 0.2 mm filtered.
Quality of BSA is critical (see Technical Hints).
Block Buffer: 1% BSA in PBS, pH 7.2-7.4, 0.2 μm filtered (R&D Systems, Catalog # DY995).
Quality of BSA is critical (see Technical Hints).
Stop Solution: 2 N H₂SO₄ (R&D Systems, Catalog # DY994).

PRECAUTIONS

Some components in this kit contain ProClin® which may cause an allergic skin reaction. Avoid breathing mist.
The Stop Solution suggested for use with this kit is an acid solution.
The Color Reagent B suggested for use with this kit may cause skin, eye, and respiratory irritation. Avoid breathing fumes.
Wear protective gloves, clothing, eye, and face protection. Wash hands thoroughly after handling. Please refer to the MSDS on our website prior to use.

CALIBRATION

This DuoSet is calibrated against a highly purified E. coli-expressed recombinant porcine TNF-α produced at R&D Systems.

MATERIALS PROVIDED & STORAGE CONDITIONS

Store the unopened kit at 2-8 °C. Do not use past kit expiration date.

<table>
<thead>
<tr>
<th>DESCRIPTION</th>
<th>PART #</th>
<th># VIALS</th>
<th>STORAGE OF OPENED/RECONSTITUTED MATERIAL</th>
</tr>
</thead>
<tbody>
<tr>
<td>Porcine TNF-α Capture Antibody</td>
<td>841019</td>
<td>1 vial</td>
<td>Refer to the lot-specific Certificate of Analysis (C of A) for storage conditions.</td>
</tr>
<tr>
<td>Porcine TNF-α Detection Antibody</td>
<td>842815</td>
<td>1 vial</td>
<td></td>
</tr>
<tr>
<td>Porcine TNF-α Standard</td>
<td>841021</td>
<td>3 vials</td>
<td></td>
</tr>
<tr>
<td>Streptavidin-HRP</td>
<td>890603</td>
<td>1 vial</td>
<td></td>
</tr>
</tbody>
</table>

REAGENT PREPARATION

Bring all reagents to room temperature before use. Allow all components to sit for a minimum of 15 minutes with gentle agitation after initial reconstitution. Working dilutions should be prepared and used immediately.

Streptavidin-HRP: 1.0 mL of streptavidin conjugated to horseradish peroxidase. Dilute to the working concentration specified on the vial label using Reagent Diluent.

Mouse Anti-Porcine TNF-α Capture Antibody: Refer to the lot-specific C of A for amount supplied. Reconstitute with 1.0 mL of PBS. Dilute in PBS without carrier protein to the working concentration indicated on the C of A.

Biotinylated Mouse Anti-Porcine TNF-α Detection Antibody: Refer to the lot-specific C of A for amount supplied. Reconstitute with 1.0 mL of Reagent Diluent. Dilute in Reagent Diluent to the working concentration indicated on the C of A.

Recombinant Porcine TNF-α Standard: Refer to the lot-specific C of A for amount supplied. Reconstitute each vial with 0.5 mL of PBS.

GENERAL ELISA PROTOCOL

Plate Preparation

1. Dilute the Capture Antibody to the working concentration in PBS without carrier protein. Immediately coat a 96-well microplate with 100 μL per well of the diluted Capture Antibody. Seal the plate and incubate overnight at room temperature.

2. Aspirate each well and wash with Wash Buffer, repeating the process two times for a total of three washes. Wash by filling each well with Wash Buffer (400 μL) using a squirt bottle, manifold dispenser, or autowasher. Complete removal of liquid at each step is essential for good performance. After the last wash, remove any remaining Wash Buffer by aspirating or by inverting the plate and blotting it against clean paper towels.

3. Block plates by adding 300 μL of Block Buffer to each well. Incubate at room temperature for a minimum of 1 hour.

4. Repeat the aspiration/wash as in step 2. The plates are now ready for sample addition.

Assay Procedure

1. Add 100 μL of sample or standards in Reagent Diluent, or an appropriate diluent, per well. Cover with an adhesive strip and incubate 2 hours at room temperature.

2. Repeat the aspiration/wash as in step 2 of Plate Preparation.

3. Add 100 μL of the Detection Antibody, diluted in Reagent Diluent, to each well. Cover with a new adhesive strip and incubate 2 hours at room temperature.

4. Repeat the aspiration/wash as in step 2 of Plate Preparation.

5. Add 100 μL of the working dilution of Streptavidin-HRP to each well. Cover the plate and incubate for 20 minutes at room temperature. Avoid placing the plate in direct light.

6. Repeat the aspiration/wash as in step 2.

7. Add 100 μL of Substrate Solution to each well. Incubate for 20 minutes at room temperature. Avoid placing the plate in direct light.

8. Add 50 μL of Stop Solution to each well. Gently tap the plate to ensure thorough mixing.

9. Determine the optical density of each well immediately, using a microplate reader set to 450 nm. If wavelength correction is available, set to 540 nm or 570 nm. If wavelength correction is not available, subtract readings at 540 nm or 570 nm from the readings at 450 nm. This subtraction will correct for optical imperfections in the plate. Readings made directly at 450 nm without correction may be higher and less accurate.

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