Human TNF-α
ELISA MAX™ Set Deluxe

Cat. No. 430204 (5 plates)
430205 (10 plates)
430206 (20 plates)

ELISA Set for Accurate Cytokine Quantification from Cell Culture Supernatant, Serum, Plasma or Other Body Fluids

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# Table of Contents

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Introduction</td>
<td>3</td>
</tr>
<tr>
<td>Intended Use</td>
<td>3</td>
</tr>
<tr>
<td>Principle of the Test</td>
<td>3</td>
</tr>
<tr>
<td>Materials Provided</td>
<td>3</td>
</tr>
<tr>
<td>Materials to Be Provided by the End-User</td>
<td>4</td>
</tr>
<tr>
<td>Storage Information</td>
<td>4</td>
</tr>
<tr>
<td>Health Hazard Warnings</td>
<td>4</td>
</tr>
<tr>
<td>Specimen Collection and Handling</td>
<td>5</td>
</tr>
<tr>
<td>Reagent Preparation</td>
<td>5</td>
</tr>
<tr>
<td>Assay Procedure</td>
<td>6</td>
</tr>
<tr>
<td>Assay Procedure Summary</td>
<td>8</td>
</tr>
<tr>
<td>Calculation of Results</td>
<td>9</td>
</tr>
<tr>
<td>Typical Data</td>
<td>9</td>
</tr>
<tr>
<td>Troubleshooting</td>
<td>10</td>
</tr>
<tr>
<td>ELISA Template</td>
<td>11</td>
</tr>
</tbody>
</table>
Introduction:

Tumor Necrosis Factor-α (TNF-α) is a potent lymphoid factor which exerts cytotoxic effects on a wide range of tumor cells and certain other target cells. Human TNF-α is a 17.4 kD protein containing 157 amino acid residues.

Intended Use:

BioLegend’s ELISA MAX™ Set Standard is specifically designed for the accurate quantification of human TNF-α from cell culture supernatant, serum, plasma or other bodily fluids. It is ready-to-use, accurate, and sensitive.

This kit is for research use only.

Principle of the Test:

BioLegend’s ELISA MAX™ Set Standard is a sandwich Enzyme-Linked Immunosorbent Assay (ELISA). A human TNF-α specific monoclonal antibody is first coated on a 96-well plate. Standards and samples are added to the wells, and TNF-α binds to the immobilized capture antibody. Next, a biotinylated anti-human TNF-α detection antibody is added, followed by avidin-horseradish peroxidase, producing an antibody-antigen-antibody “sandwich”. TMB Substrate Solution is subsequently added, producing a blue color in proportion to the amount of TNF-α present in the sample. Finally, the Stop Solution changes the reaction color from blue to yellow, and the microwell absorbances are read at 450 nm.

Materials Provided:

1. Human TNF-α ELISA Capture Antibody
2. Human TNF-α ELISA Detection Antibody
3. Human TNF-α Standard
4. Avidin-HRP
5. Substrate Solution A & B
6. Coating Buffer A (5X)
7. Assay Diluent (5X)
8. NUNC Maxisorp 96-well ELISA plates
9. Human TNF-α Lot Specifications/Analysis Certificate
Materials to Be Provided by the End-User

- Microplate Reader able to measure absorbance at 450 nm
- Adjustable pipettes to measure volumes ranging from 10 µl to 25 ml
- Deionized (DI) water
- Wash Buffer (Phosphate-Buffered Saline (PBS) + 0.05% Tween-20, pH=7.4)
- Wash bottle or automated microplate washer
- Stop Solution (2 N H₂SO₄)
- Log-Log graph paper or software for data analysis
- Tubes to prepare standard dilutions
- Timer
- Absorbent paper

Storage Information

1. Store kit components at 4° C.

2. After reconstitution of the lyophilized standard with 0.2 ml of 1X Assay Diluent, aliquot into polypropylene vials and store at -70°C. Do not repeatedly freeze/thaw the recombinant protein standard as loss of activity may occur.

3. Prior to use, bring all components to room temperature (18-25° C). Upon assay completion return all components to appropriate storage conditions.

Health Hazard Warnings

1. Reagents that contain preservatives may be harmful if ingested, inhaled or absorbed through the skin. Refer to the MSDS online for details (www.biolegend.com).

2. TMB Substrate Solution is harmful if inhaled or ingested. Additionally, avoid skin, eye or clothing contact with the Substrate Reagents.

3. To reduce the likelihood of blood-borne transmission of infectious agents, handle all serum and/or plasma in accordance with NCCLS regulations.
Specimen Collection and Handling

*Cell Culture Supernatant:* If necessary, centrifuge to remove debris prior to analysis. Samples can be stored at ≤ -20°C. Avoid repeated freeze/thaw cycles.

*Serum:* Use a serum separator tube and allow clotting for at least 30 minutes, then centrifuge for 10 minutes at 1000X g. Remove serum layer and assay immediately or store serum samples at ≤ -20°C. Avoid repeated freeze/thaw cycles. Serum specimens should be clear and non-hemolyzed.

*Plasma:* Collect blood sample in a citrate, heparin or EDTA containing tube. Centrifuge for 10 minutes at 1000 X g within 30 minutes of collection. Assay immediately or store plasma samples at ≤ -20°C. Avoid repeated freeze/thaw cycles. Plasma specimens should be clear and non-hemolyzed.

Reagent Preparation

*Do not use sodium azide in any solutions as it inhibits the activity of the horseradish-peroxidase enzyme. All reagents should be diluted immediately prior to use; see Lot Specifications/Analysis Certificate for dilution instructions.*

1. Dilute 5X Coating Buffer to 1X with DI water.
2. Dilute pre-titrated Capture Antibody 1:200 in 1X Coating Buffer.
3. Dilute 5X Assay Diluent to 1X with DI water.
4. Lyophilized vials are under vacuum pressure. Reconstitute lyophilized standard with 0.2 ml of 1X Assay Diluent, re-cap vial, and mix well. Allow the reconstituted standard to sit for 15-20 minutes at room temperature, then invert/vortex to mix. Aliquot remaining standard into polypropylene vials and store at -70°C.
5. Dilute pre-titrated Biotinylated Detection Antibody 1:200 in 1X Assay Diluent.
6. Dilute Avidin-HRP 1:1,000 in 1X Assay Diluent.
7. TMB Substrate Solution is the mixture of equal volumes of Substrate Solution A with Substrate Solution B. For example, for one plate mix 6 ml Substrate Solution A with 6 ml of Substrate Solution B in a clean container (solution should be clear and colorless). Mix immediately before use.
8. Wash Buffer: PBS with 0.05% Tween-20. PBS: 8.0 g NaCl, 1.16 g Na₂HPO₄, 0.2 g KH₂PO₄, 0.2 g KCl, add deionized water to 1 L; pH to 7.4. Add 0.5 mL of Tween-20, mix.
Assay Procedure

Do not mix reagents from different kits or lots. Reagents and/or antibodies from different manufacturers should not be used with this set.

1. One day prior to running the ELISA, dilute Capture Antibody in 1X Coating Buffer as described in Reagent Preparation. Add 100 µl of this Capture Antibody solution to all wells of a 96-well plate provided in this kit. Seal plate and incubate overnight (16-18 hrs) at 4° C.

2. Bring all reagents to room temperature prior to use. It is strongly recommended that all standards and samples be run in duplicate or triplicate. A standard curve is required for each assay.

3. Wash plate 4 times with at least 300 µl Wash Buffer per well and blot residual buffer by firmly tapping plate upside down on absorbent paper. All subsequent washes should be performed similarly.

4. To block non-specific binding and reduce background, add 200 µl 1X Assay Diluent per well.

5. Seal plate and incubate at room temperature for 1 hour with shaking at 200 rpm on a plate shaker*.

6. While plate is being blocked, prepare appropriate sample dilutions (if necessary) and standards.

7. Prepare 1 ml of top standard in Assay Diluent at 500 pg/ml from stock solution in Assay Diluent (refer to Lot-Specifications/Analysis Certificate). Perform six two-fold serial dilutions of the 500 pg/ml top standard with Assay Diluent in separate tubes. After diluting, the human TNF-α standard concentrations are 500 pg/ml, 250 pg/ml, 125 pg/ml, 62.5 pg/ml, 31.3 pg/ml, 15.6 pg/ml, and 7.8 pg/ml. Assay Diluent serves as the zero standard (0 pg/ml).

8. Wash plate 4 times with Wash Buffer as in step 3.

9. Add 100µl/well of standards or samples to the appropriate wells. If dilution is required, samples should be diluted in 1X Assay Diluents before adding to the wells.

10. Seal plate and incubate at room temperature for 2 hours with shaking*.

11. Wash plate 4 times with Wash Buffer as described in step 3.

12. Add 100 µl of diluted Detection Antibody solution to each well, seal plate and incubate at room temperature for 1 hour with shaking*.

13. Wash plate 4 times with Wash Buffer as in step 3.
BioLegend ELISA MAX™ Set Standard

14. Add 100 µl of diluted Avidin-HRP solution to each well, seal plate and incubate at room temperature for 30 minutes with shaking*.

15. Wash plate 4 times with Wash Buffer as in step 3. For this final wash, soak wells in Wash Buffer for 30 seconds to 1 minute for each wash. This will help minimize background.

16. Add 100 µl of freshly mixed TMB Substrate Solution and incubate in the dark for 15 minutes with shaking*. Positive wells should turn a bluish color. It is not necessary to seal the plate during this step.

17. Stop reaction by adding 100 µl of Stop Solution to each well. Positive wells should turn from blue to yellow.

18. Read absorbance at 450 nm within 30 minutes. If the reader can read at 570 nm, the absorbance at 570 nm can be subtracted from the absorbance at 450 nm.

*NOTE: Although shaking is not required during any incubation steps, it may increase assay sensitivity.
BioLegend ELISA MAX™ Set Standard

Assay Procedure Summary

1. Coat plate with 100μl diluted Capture Antibody
   Incubate overnight, 4°C

2. Wash 4 x
   Add 200μl Assay Diluent
   Incubate 1 hr, RT, shaking

3. Wash 4 x
   Add 100μl diluted standards and samples
   Incubate 2 hr, RT, shaking

4. Wash 4 x
   Add 100μl Biotinylated Detection Antibody
   Incubate 1 hr, RT, shaking

5. Wash 4 x
   Add 100μl Avidin-HRP
   Incubate 30’, RT, shaking

6. Wash 5 x
   Add 100μl Substrate Solution
   Incubate 15’, RT, shaking

7. Add 100μl Stop Solution

8. Read absorbance at 450nm and 570nm
Calculation of Results

Determine the mean absorbance for each set of duplicate or triplicate standards, controls and samples. Plot the standard curve on log-log graph paper, with cytokine concentration on the x-axis and absorbance on the y-axis. Draw a best fit straight line through the standard points. To determine the unknown cytokine concentrations, find mean absorbance value of unknowns 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 cytokine concentration. If samples were diluted, multiply by the appropriate dilution factor. The data can be best calculated with computer-based curve-fitting software using a 5- or 4-parameter logistics curve-fitting algorithm. If a test sample’s O.D. value falls outside the linear portion of the standard curve, that test sample needs to be reanalyzed at a higher (or lower) dilution as appropriate.

Typical Data

**Standard Curve**: This standard curve was generated at BioLegend for demonstration purposes only. A standard curve must be run with each assay.

![Standard Curve Graph](image)

**Performance Characteristics:**

*Sensitivity*: The minimum detectable concentration of TNF-α is 8 pg/ml.

*Specificity*: No cross reactivity was observed when this kit was used to analyze multiple human, mouse and rat recombinant proteins other than human TNF-α.
Troubleshooting

**Poor Precision**
- Inadequate washing of wells
- Inadequate mixing of reagents
- Improper/inaccurate pipetting
- Improper plate sealing

**Poor Signal**
- Avoid sodium azide in wash buffers, as sodium azide inhibits the enzymatic activity of HRP.
- Verify that appropriate antibody pairs were used.
- Inadequate reagent volumes added to wells
- Incorrect incubation times and/or temperature

**Poor Standard Curve**
- Do not leave recombinant proteins at room temperature. If necessary, store recombinant proteins at 4°C for up to 8 hours prior to use.
- Recombinant protein vials should be quick-spun for maximum recovery.
- Incomplete washing of wells
- Improper/inaccurate pipetting

**High Background**
- Increase stringency of washing steps by soaking plates for ~1 minute during washes prior to TMB Substrate Solution addition.
- TMB Substrate Solution should be clear and colorless prior to addition to wells. Ensure that substrate was prepared in a clean container immediately prior to use.
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<thead>
<tr>
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<th>2</th>
<th>3</th>
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<th>5</th>
<th>6</th>
<th>7</th>
<th>8</th>
<th>9</th>
<th>10</th>
<th>11</th>
<th>12</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
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