Quantikine®

Human TNF-β/TNFSF1B Immunoassay

Catalog Number DTB00

For the quantitative determination of human tumor necrosis factor beta (TNF-β) concentrations in cell culture supernates.

This package insert must be read in its entirety before using this product.

FOR RESEARCH USE ONLY.
NOT FOR USE IN DIAGNOSTIC PROCEDURES.

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INTRODUCTION

Tumor necrosis factor beta (TNF-β), also known as lymphotoxin alpha, and tumor necrosis factor alpha (TNF-α), also known as cachectin, are two closely related proteins (approximately 34% amino acid sequence identity) that bind to the same cell surface receptors and show many common biological functions. TNF-β plays a critical role in lymphoid development and in normal host resistance to infection and to the growth of malignant tumors, serving as an immunostimulant and as a mediator of the inflammatory response. Overproduction of TNF-β has been implicated as playing a role in a number of autoimmune disorders, including multiple sclerosis and insulin-dependent diabetes mellitus. TNF-β is produced by activated T and B cells, NK cells, astrocytes, and human myeloma cells (1 - 5).

Human TNF-β cDNA encodes a 205 amino acid (aa) residue precursor protein with a 34 aa residue signal peptide that is cleaved to generate the mature secreted protein (6). The biologically active secreted TNF-β exists as a homotrimer (7, 8). It has been shown that TNF-β is also present on the cell surface of activated T, B, and LAK cells as a heterotrimeric complex with lymphotoxin beta (LT-β), a type II membrane protein that is another member of the TNF ligand family (6). The genes for TNF-α, TNF-β and LT-β are closely linked within the major histocompatibility complex on human chromosome 6 (6).

Two distinct TNF receptors, referred to as type I (type B or p55) and type II (type A or p75), that specifically bind TNF-α and TNF-β with equal affinity have been identified (9, 10). The two TNF receptors transduce signals independently of one another. The amino acid sequence of the extracellular domains of the two receptors are homologous and both receptors are members of the TNF receptor family which also includes the NGF receptor, *fas* antigen, CD27, CD30, and CD40. The intracellular domains of the two receptors are apparently unrelated, suggesting that the two receptors employ different signal transduction pathways. Soluble forms of both types of receptors have been found in human serum and urine (11 - 13). These soluble receptors are capable of neutralizing the biological activities of the TNFs and may serve to modulate the activities of TNF.

The Quantikine Human TNF-β Immunoassay is a 4.5 hour solid phase ELISA designed to measure TNF-β levels in cell culture supernates. It contains *E. coli*-expressed recombinant human TNF-β and antibodies raised against the recombinant factor. This immunoassay has been shown to quantitate the recombinant human TNF-β accurately. Results obtained using natural human TNF-β showed dose curves that were parallel to the standard curves obtained using the recombinant Quantikine kit standards. These results indicate that the Quantikine Human TNF-β Immunoassay kit can be used to determine relative mass values for natural human TNF-β.

PRINCIPLE OF THE ASSAY

This assay employs the quantitative sandwich enzyme immunoassay technique. A monoclonal antibody specific for TNF-β has been pre-coated onto a microplate. Standards and samples are pipetted into the wells and any TNF-β present is bound by the immobilized antibody. After washing away any unbound substances, an enzyme-linked polyclonal antibody specific for TNF-β is added to the wells. Following a wash to remove any unbound antibody-enzyme reagent, a substrate solution is added to the wells and color develops in proportion to the amount of TNF-β bound in the initial step. The color development is stopped and the intensity of the color is measured.

LIMITATIONS OF THE PROCEDURE

- FOR RESEARCH USE ONLY. NOT FOR USE IN DIAGNOSTIC PROCEDURES.
- The kit should not be used beyond the expiration date on the kit label.
- Do not mix or substitute reagents with other lots or sources.
- It is important that the Calibrator Diluent selected for the standard curve be consistent with the samples being assayed.
- If samples generate values higher than the highest standard, dilute the samples with Calibrator Diluent and repeat the assay.
- Any variation in standard diluent, operator, pipetting technique, washing technique, incubation time or temperature, and kit age can cause variation in binding.
- This assay is designed to eliminate inteference by soluble receptors, binding proteins, and other factors present in biological samples. Until all factors have been tested in the Quantikine immunoassay, the possibility of interference cannot be excluded.

MATERIALS PROVIDED

TNF-β Microplate (Part 890033) - 96 well polystyrene microplate (12 strips of 8 wells) coated with a mouse monoclonal antibody against TNF-β.

TNF-β Conjugate (Part 890034) - 21 mL of polyclonal antibody against TNF-β conjugated to horseradish peroxidase, with preservatives.

TNF-β Standard (Part 890035) - 50 ng of recombinant human TNF-β in a buffered protein base with preservatives, lyophilized.

Calibrator Diluent RD5-5 (Part 895485) - 21 mL of a buffered protein base with preservatives.

Wash Buffer Concentrate (Part 895003) - 21 mL of a 25-fold concentrated solution of buffered surfactant with preservatives.

Color Reagent A (Part 895000) - 12.5 mL of stabilized hydrogen peroxide.

Color Reagent B (Part 895001) - 12.5 mL of stabilized chromogen (tetramethylbenzidine).

Stop Solution (Part 895032) - 6 mL of 2 N sulfuric acid.

Plate Covers - 4 adhesive strips.

STORAGE

<table>
<tr><td>Unopened Kit</td><td colspan="2">Store at 2 - 8° C. Do not use past kit expiration date.</td></tr>
<tr><td rowspan="8">Opened/ Reconstituted Reagents</td><td>Diluted Wash Buffer</td><td rowspan="6">May be stored for up to 1 month at 2 - 8° C.*</td></tr>
<tr><td>Stop Solution</td></tr>
<tr><td>Calibrator Diluent RD5-5</td></tr>
<tr><td>Conjugate</td></tr>
<tr><td>Unmixed Color Reagent A</td></tr>
<tr><td>Unmixed Color Reagent B</td></tr>
<tr><td>Standard</td><td>Aliquot and store for up to 1 month at ≤ -20° C in a manual defrost freezer.* Avoid repeated freeze-thaw cycles.</td></tr>
<tr><td>Microplate Wells</td><td>Return unused wells to the foil pouch containing the desiccant pack, reseal along entire edge of zip-seal. May be stored for up to 1 month at 2 - 8° C.*</td></tr>
</table>

*Provided this is within the expiration date of the kit.

OTHER SUPPLIES REQUIRED

- Microplate reader capable of measuring absorbance at 450 nm, with the correction wavelength set at 540 nm or 570 nm.
- Pipettes and pipette tips.
- Deionized or distilled water.
- Squirt bottle, manifold dispenser, or automated microplate washer.
- 500 mL graduated cylinder.
- Human TNF-β Controls (optional; available from R&D Systems).

PRECAUTION

The Stop Solution provided with this kit is an acid solution. Wear eye, hand, face, and clothing protection when using this material.

SAMPLE COLLECTION AND STORAGE

Cell Culture Supernates - Remove particulates by centrifugation and assay immediately or aliquot and store samples at ≤ -20° C. Avoid repeated freeze-thaw cycles.

REAGENT PREPARATION

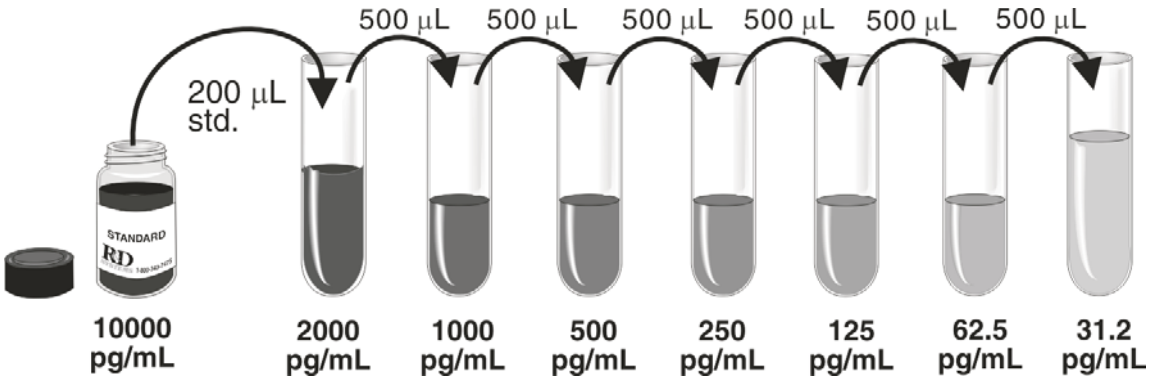
Bring all reagents to room temperature before use.

Wash Buffer - If crystals have formed in the concentrate, warm to room temperature and mix gently until the crystals have completely dissolved. Dilute 20 mL of Wash Buffer Concentrate into deionized or distilled water to prepare 500 mL of Wash Buffer.

Substrate Solution - Color Reagents A and B should be mixed together in equal volumes within 15 minutes of use. Protect from light. 200 µL of the resultant mixture is required per well.

TNF-β Standard - Reconstitute the TNF-β Standard with 5.0 mL of Calibrator Diluent RD5-5. This reconstitution produces a stock solution of 10,000 pg/mL. Allow the standard to sit for a minimum of 15 minutes with gentle agitation prior to making dilutions.

Pipette 800 µL of Calibrator Diluent RD5-5 into the 2000 pg/mL tube and 500 µL into each remaining tube. Use the stock solution to produce a dilution series (below). Mix each tube thoroughly before the next transfer. The 2000 pg/mL dilution serves as the high standard. Calibrator Diluent RD5-5 serves as the zero standard (0 pg/mL).



ASSAY PROCEDURE

Bring all reagents and samples to room temperature before use. It is recommended that all samples, standards, and controls be assayed in duplicate.

1. Prepare all reagents, samples, and working standards as directed in the previous sections.

2. Remove excess microplate strips from the plate frame, return them to the foil pouch containing the desiccant pack, reseal.

3. Add 200 µL of Standard, control, or sample per well. Cover with the adhesive strip provided. Incubate for 2 hours at room temperature.

4. Aspirate each well and wash, repeating the process three times for a total of four 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 to good performance. After the last wash, remove any remaining Wash Buffer by aspirating or decanting. Invert the plate and blot it against clean paper towels.

5. Add 200 µL of TNF-β Conjugate to each well. Cover with a new adhesive strip. Incubate for 2 hours at room temperature.

6. Repeat the aspiration/wash as in step 4.

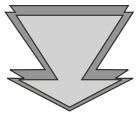
7. Add 200 µL of Substrate Solution to each well. Incubate for 20 minutes at room temperature. **Protect from light.**

8. Add 50 µL of Stop Solution to each well. The color in the wells should change from blue to yellow. If the color in the wells is green or the color change does not appear uniform, gently tap the plate to ensure thorough mixing.

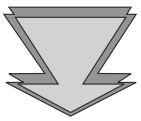
9. Determine the optical density of each well within 30 minutes, using a microplate reader set to 450 nm. If wavelength correction is available, set to 540 nm or 570 nm. If wavelength correction is not available, subtract readings at 540 nm or 570 nm from the readings at 450 nm. This subtraction will correct for optical imperfections in the plate. Readings made directly at 450 nm without correction may be higher and less accurate.

ASSAY PROCEDURE SUMMARY

1. Prepare reagents and standards as instructed.

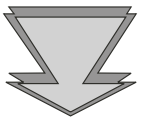


2. Add 200 µL Standard, control or sample to each well. Incubate 2 hours at RT.

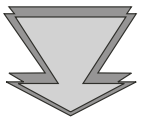


3. Aspirate and wash 4 times.

4. Add 200 µL Conjugate to each well. Incubate 2 hours at RT.



5. Aspirate and wash 4 times.



6. Add 200 µL Substrate Solution to each well. **Protect from light.** Incubate 20 min. RT.

7. Add 50 µL Stop Solution to each well. Read at 450 nm within 30 min.
λ correction 540 or 570 nm

CALCULATION OF RESULTS

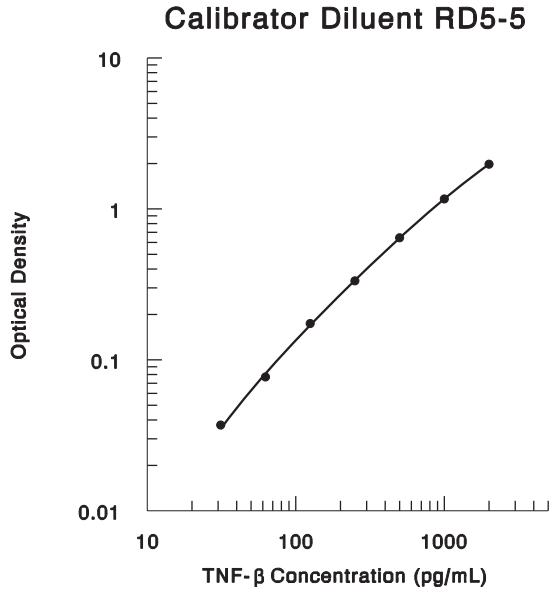
Average the duplicate readings for each standard, control, and sample and subtract the average zero standard optical density.

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 concentration 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.

TYPICAL DATA

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



(pg/mL)	O.D.	Average	Corrected
0	0.020 0.021	0.020	—
31.2	0.058 0.057	0.058	0.038
62.5	0.101 0.094	0.098	0.078
125	0.204 0.185	0.194	0.174
250	0.355 0.353	0.354	0.334
500	0.666 0.663	0.664	0.644
1000	1.222 1.148	1.185	1.165
2000	2.052 1.946	1.999	1.979

TECHNICAL HINTS

- When mixing or reconstituting protein solutions, always avoid foaming.
- To avoid cross-contamination, change pipette tips between additions of each standard level, between sample additions, and between reagent additions. Also, use separate reservoirs for each reagent.
- When using an automated plate washer, adding a 30 second soak period following the addition of wash buffer, and/or rotating the plate 180 degrees between wash steps may improve assay precision.
- To ensure accurate results, proper adhesion of plate sealers during incubation steps is necessary.
- Substrate Solution should remain colorless until added to the plate. Keep Substrate Solution protected from light. Substrate Solution should change from colorless to gradations of blue.
- Stop Solution should be added to the plate in the same order as the Substrate Solution. The color developed in the wells will turn from blue to yellow upon addition of the Stop Solution. Wells that are green in color indicate that the Stop Solution has not mixed thoroughly with the Substrate Solution.

PRECISION

Intra-assay Precision (Precision within an assay)
Three samples of known concentration were tested twenty times on one plate to assess intra-assay precision.

Inter-assay Precision (Precision between assays)
Three samples of known concentration were tested in twenty separate assays to assess inter-assay precision.

	Intra-assay Precision			Inter-assay Precision		
Sample	1	2	3	1	2	3
n	20	20	20	20	20	20
Mean (pg/mL)	104	383	676	119	570	1222
Standard deviation	2.8	10.0	24.3	11.0	44.0	107
CV (%)	2.7	2.6	3.6	9.2	7.7	8.8

RECOVERY

The recovery of TNF-β spiked to four different levels in eight cell culture media samples throughout the range of the assay was evaluated.

Sample Type	Average % Recovery	Range
Cell culture media	91	81 - 102%

SENSITIVITY

The minimum detectable dose of TNF-β is typically less than 7 pg/mL.

The minimum detectable dose was determined by adding two standard deviations to the mean optical density value of twenty zero standard replicates and calculating the corresponding concentration.

CALIBRATION

This assay has been calibrated to the NIBSC/WHO recombinant human TNF-β interim reference preparation 87/640.

SPECIFICITY

This assay recognizes both natural and recombinant human TNF-β. The factors listed below were prepared at 10 ng/mL in Calibrator Diluent and assayed for cross-reactivity. No significant cross-reactivity was observed.

Recombinant human:	**Other proteins:**
GM-CSF	bovine FGF acidic
IL-1α	human PDGF
IL-1β	porcine PDGF
IL-2	human TGF-β1
IL-3	porcine TGF-β1
IL-4	porcine TGF-β1.2
IL-6	porcine TGF-β2
IL-8	
TNF-α	

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