Quantikine®

Rat TNF-α/TNFSF1A Immunoassay

Catalog Number RTA00

For the quantitative determination of rat tumor necrosis factor alpha (TNF-α) concentrations in cell culture supernates, rat serum and plasma.

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 alpha (TNF-α), also known as cachectin; and tumor necrosis factor beta (TNF-β), also known as lymphotoxin, 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-α and -β play critical roles in normal host resistance to infection and to the growth of malignant tumors, serving as immunostimulants and as mediators of the inflammatory response. Over-production of TNFs, however, has been implicated as playing a role in a number of pathological conditions, including cachexia, septic shock, and autoimmune disorders. TNF-α is produced by activated macrophages and other cell types including T and B cells, NK cells, LAK cells, astrocytes, endothelial cells, smooth muscle cells and some tumor cells (1 - 4).

Rat TNF-α cDNA encodes a 235 amino acid (aa) residue type II membrane protein (5). The 156 aa residue soluble TNF-α is released from the C-terminus of the membrane-anchored TNF-α by TNF-α-converting enzyme (TACE), a matrix metalloprotease (6, 7). The membrane-anchored form of TNF-α has been shown to have lytic activity and may also play an important role in intercellular communication (8). The biologically active TNF-α has been shown to exist as a trimer (9, 10).

Two distinct TNF receptors, referred to as type I (or type B or p55) and type II (or type A or p75), that specifically bind TNF-α and TNF-β with equal affinity have been identified (11, 12). 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 include 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 (13 - 15). These soluble receptors are capable of neutralizing the biological activities of the TNFs and may serve to modulate the activities of TNF.

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

PRINCIPLE OF THE ASSAY

This assay employs the quantitative sandwich enzyme immunoassay technique. A monoclonal antibody specific for rat TNF-α has been pre-coated onto a microplate. Standards, Controls, and samples are pipetted into the wells and any rat TNF-α present is bound by the immobilized antibody. After washing away any unbound substances, an enzyme-linked polyclonal antibody specific for rat TNF-α is added to the wells. Following a wash to remove any unbound antibody-enzyme reagent, a substrate solution is added to the wells. The enzyme reaction yields a blue product that turns yellow when the Stop Solution is added. The intensity of the color measured is in proportion to the amount of rat TNF-α bound in the initial step. The sample values are then read off the standard curve.

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 those from other lots or sources.
- If samples generate values higher than the highest standard, further dilute the samples with Calibrator Diluent and repeat the assay.
- Any variation in operator, pipetting technique, washing technique, incubation time or temperature, and kit age can cause variation in binding.
- This assay is designed to eliminate interference 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.

PRECAUTION

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

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.
- For best results, pipette reagents and samples into the center of each well.
- It is recommended that the samples be pipetted within 15 minutes.
- 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. The color developed in the wells will turn from blue to yellow upon addition of the Stop Solution.
- Stop Solution should be added to the plate in the same order as the Substrate Solution.

REAGENTS

Rat TNF-α Microplates (Part 890682) - Two 96 well polystyrene microplates (12 strips of 8 wells) coated with a monoclonal antibody specific for rat TNF-α.

Rat TNF-α Conjugate (Part 892668) - 23 mL of a polyclonal antibody against rat TNF-α conjugated to horseradish peroxidase, with preservatives.

Rat TNF-α Standard (Part 890684) - 3 vials (1.6 ng/vial) of recombinant rat TNF-α in a buffered protein base with preservatives, lyophilized.

Rat TNF-α Control (Part 890685) - 3 vials of recombinant rat TNF-α in a buffered protein base with preservatives, lyophilized. The concentration range of rat TNF-α after reconstitution is shown on the vial label. The assay value of the Control should be within the range specified on the Control label.

Assay Diluent RD1-41 (Part 895514) - 12.5 mL of a buffered protein base, with preservatives.

Calibrator Diluent RD5-17 (Part 895512) - 2 vials (21 mL/vial) of a buffered protein base, with preservatives.

Wash Buffer Concentrate (Part 895024) - 50 mL of a 25-fold concentrated solution of a buffered surfactant, with preservative.

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 895174) - 23 mL of a diluted hydrochloric acid solution.

Plate Covers (Part 640197) - 8 adhesive plate sealers.

STORAGE

<table>
<tr><td>Unopened Kit</td><td colspan="2">Store at 2 - 8° C. Do not use beyond kit expiration date.</td></tr>
<tr><td rowspan="10">Opened/ Reconstituted Reagents</td><td>Diluted Wash Buffer</td><td rowspan="7">May be stored for up to 1 month at 2 - 8° C.*</td></tr>
<tr><td>Stop Solution</td></tr>
<tr><td>Calibrator Diluent RD5-17</td></tr>
<tr><td>Assay Diluent RD1-41</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>Rat TNF-α Standard (800 pg/mL)</td><td rowspan="2">Use a new Standard and Control for each assay.</td></tr>
<tr><td>Rat TNF-α Control</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.
- 100 mL and 1000 mL graduated cylinders.
- **Polypropylene tubes.**

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.

Serum - Allow blood samples to clot for 2 hours at room temperature or overnight at 2 - 8° C before centrifuging for 20 minutes at approximately 1000 x g. Remove serum and assay immediately or aliquot and store samples at ≤ -20° C. Avoid repeated freeze-thaw cycles.

Plasma - Collect plasma using EDTA or heparin as an anticoagulant. Centrifuge for 20 minutes at approximately 1000 x g within 30 minutes of collection. Assay immediately or aliquot and store samples at ≤ -20° C. Avoid repeated freeze-thaw cycles.

Note: *Grossly hemolyzed or lipemic samples may not be suitable for measurement of rat TNF-α with this assay.*

SAMPLE PREPARATION

Rat serum and plasma samples require a 2-fold dilution into Calibrator Diluent RD5-17 prior to assay. A suggested 2-fold dilution is 75 µL sample + 75 µL Calibrator Diluent RD5-17. Mix well.

Rat cell culture supernate samples require a 3-fold dilution into Calibrator Diluent RD5-17 prior to assay. A suggested 3-fold dilution is 50 µL sample + 100 µL Calibrator Diluent RD5-17. Mix well.

REAGENT PREPARATION

Bring all reagents to room temperature before use.

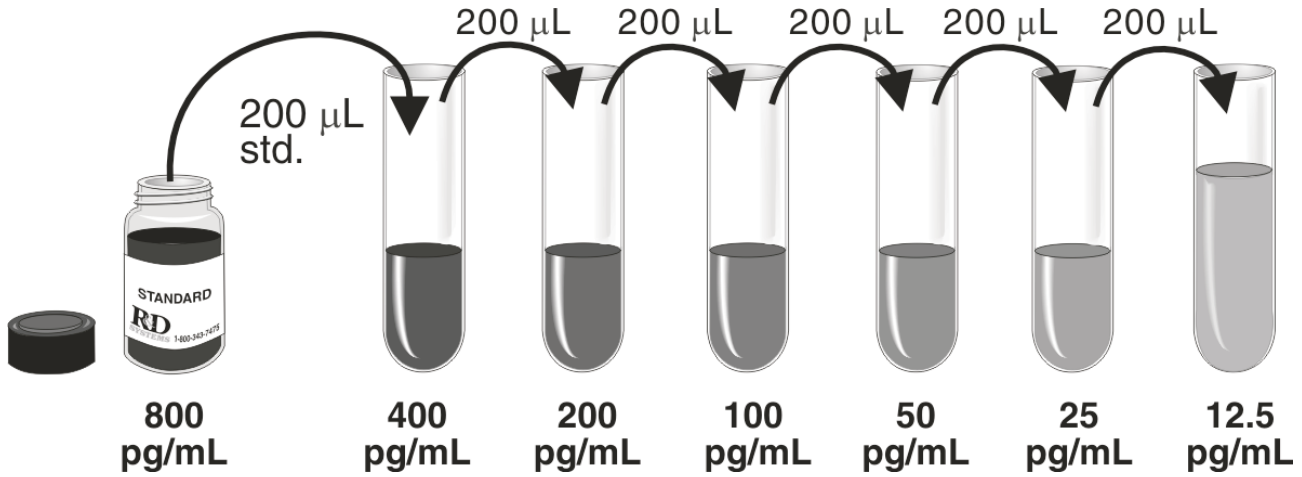
Rat TNF-α Kit Control - Reconstitute the Kit Control with 1.0 mL deionized or distilled water. Assay the Control undiluted.

Wash Buffer - If crystals have formed in the concentrate, warm to room temperature and mix gently until the crystals have completely dissolved. To prepare enough Wash Buffer for one plate, add 25 mL Wash Buffer Concentrate into deionized or distilled water to prepare 625 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. 100 µL of the resultant mixture is required per well.

Rat TNF-α Standard - Reconstitute the rat TNF-α Standard with 2.0 mL of Calibrator Diluent RD5-17. Do not substitute other diluents. This reconstitution produces a stock solution of 800 pg/mL. Allow the standard to sit for a minimum of 5 minutes with gentle mixing prior to making dilutions.

Use polypropylene tubes. Pipette 200 µL of Calibrator Diluent RD5-17 into each tube. Use the stock solution to produce a dilution series (below). Mix each tube thoroughly before the next transfer. The undiluted rat TNF-α Standard serves as the high standard (800 pg/mL). Calibrator Diluent RD5-17 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 reagents, working standards, control and samples 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 50 µL of Assay Diluent RD1-41 to the center of each well.

4. Add 50 µL of Standard, Control or sample* to the center of each well. Mix by gently tapping the plate frame for 1 minute. Cover with the adhesive strip provided. Incubate for 2 hours at room temperature. A plate layout is provided to record standards and samples assayed.

5. Aspirate each well and wash, repeating the process four times for a total of five 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 by inverting the plate and blotting it against clean paper towels.

6. Add 100 µL of rat TNF-α Conjugate to each well. Cover with a new adhesive strip. Incubate for 2 hours at room temperature.

7. Repeat the aspiration/wash as in step 5.

8. Add 100 µL of Substrate Solution to each well. Incubate for 30 minutes at room temperature. **Protect from light.**

9. Add 100 µL of Stop Solution to each well. Gently tap the plate to ensure thorough mixing.

10. 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.

*Samples require dilution as directed in the Sample Preparation section.

PROCEDURE SUMMARY AND CHECKLIST

1. ☐ Bring all reagents to room temperature.
☐ Prepare reagents, standards and samples as instructed.
☐ Return unused components to storage temperature as indicated in the instructions.

2. ☐ Add 50 µL Assay Diluent to each well.

3. ☐ Add 50 µL Standard, Control, or sample* to each well.
☐ Tap plate gently for one minute.
☐ Cover the plate and incubate for 2 hours at room temperature.

4. ☐ Aspirate and wash each well five times.

5. ☐ Add 100 µL Conjugate to each well.
☐ Cover the plate and incubate for 2 hours at room temperature.

6. ☐ Aspirate and wash each well five times.

7. ☐ Add 100 µL Substrate Solution to each well. Incubate for 30 minutes at room temperature. **Protect from light.**

8. ☐ Add 100 µL Stop Solution to each well.

9. ☐ Read Optical Density at 450 nm (correction wavelength set at 540 nm or 570 nm).

*Samples require dilution as directed in the Sample Preparation section.

CALCULATION OF RESULTS

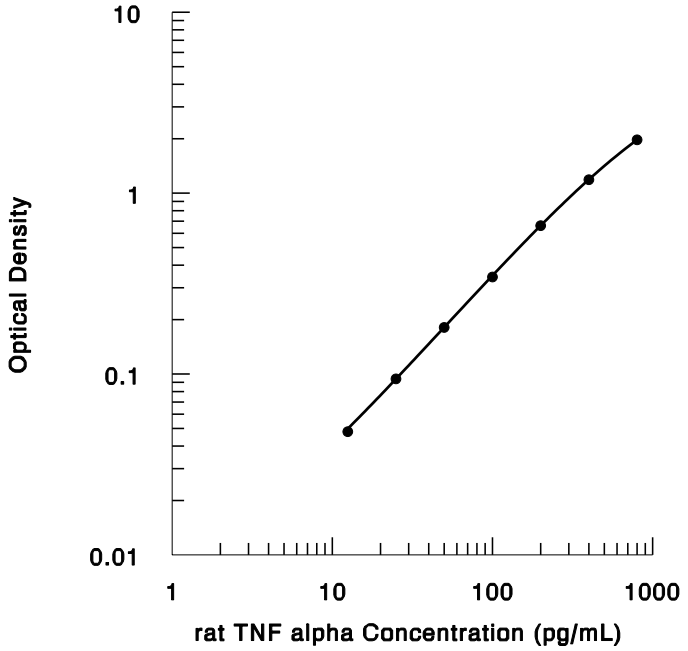
Average the duplicate readings for each standard, control, and sample and subtract the average zero standard optical density.

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 rat 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.

Because rat serum, plasma and culture supernate 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	0.034 0.034	0.034	—
12.5	0.085 0.080	0.082	0.048
25	0.128 0.127	0.128	0.094
50	0.214 0.216	0.215	0.181
100	0.383 0.372	0.378	0.344
200	0.692 0.698	0.695	0.661
400	1.218 1.222	1.220	1.186
800	2.023 1.988	2.006	1.972

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 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)	65	232	593	63	246	656
Standard deviation	3.3	5.1	12.5	6.1	23.6	57.6
CV (%)	5.1	2.2	2.1	9.7	9.6	8.8

RECOVERY

The recovery of rat TNF-α spiked to three levels throughout the range of the assay in various matrices was evaluated.

Sample Type	Average % Recovery	Range
Cell culture supernates* (n=6)	101	93 - 119%
Rat serum* (n=8)	93	83 - 105%
Rat EDTA plasma* (n=5)	96	88 - 104%
Rat heparin plasma* (n=4)	80	73 - 84%

*Samples were diluted as directed in the Sample Preparation section.

LINEARITY

To assess the linearity of the assay, four or more samples spiked with various concentrations of rat TNF-α in each matrix were diluted with Calibrator Diluent RD5-17 and then assayed. Results from typical sample dilutions are shown.

Sample	Dilution	Observed (pg/mL)	Expected (pg/mL)	$\frac{\text{Observed}}{\text{Expected}} \times 100$
Cell Culture Supernates*	spiked	620		
	1/2	292	310	94
	1/4	146	155	94
	1/8	73	78	94
	1/16	35	39	90
Rat Serum*	spiked	632		
	1/2	324	316	103
	1/4	165	158	104
	1/8	80	79	101
	1/16	39	40	98
Rat EDTA Plasma*	spiked	721		
	1/2	363	361	101
	1/4	168	180	93
	1/8	80	90	89
	1/16	42	45	93
Rat Heparin Plasma*	spiked	588		
	1/2	317	294	108
	1/4	160	147	109
	1/8	78	74	105
	1/16	38	37	103

*Samples were first diluted 3-fold and 2-fold, respectively, prior to assay, as directed in the Sample Preparation section.

SENSITIVITY

The minimum detectable dose of rat TNF-α is typically less than 5 pg/mL.

The minimum detectable dose was determined by adding two standard deviations to the mean optical density value of 20 zero standard replicates and calculating the corresponding concentration.

CALIBRATION

This immunoassay is calibrated against a highly purified *E. coli*-expressed recombinant rat TNF-α produced at R&D Systems. The recombinant N-methionyl form of rat TNF-α contains 157 amino acid residues and has a predicted molecular mass of 17 kDa.

Based on total amino acid analysis, the absorbance of a 1 mg/mL solution of the *E. coli*-expressed recombinant rat TNF-α at 280 nm was determined to be 1.33 A.U.

SAMPLE VALUES

Serum - Forty individual rat serum samples and thirteen individual rat plasma samples were evaluated for detectable levels of rat TNF-α in this assay. All samples measured less than the lowest rat TNF-α standard, 12.5 pg/mL.

Cell Culture Supernates - Rat splenocytes (1 x 10^7 cells/mL) were cultured for 2 days in DMEM supplemented with 10% fetal calf serum and stimulated with 5 μg/mL Conconavalin A. The culture supernate was assayed for rat TNF-α and measured 900 pg/mL.

SPECIFICITY

This assay recognizes both recombinant and natural rat TNF-α. The factors listed below were prepared at 50 ng/mL in Calibrator Diluent RD5-17 and assayed for cross-reactivity. Preparations of the following factors prepared at 50 ng/mL in a mid-range rat TNF-α control were assayed for interference. No significant cross-reactivity or interference was observed.

Recombinant human:
TNF-α
TNF-β
TNF sRI
TNF sRII

Recombinant rat:
IL-1β
IL-2
IL-4
CINC-1
GDNF
IFN-γ
PDGF-BB
β-NGF

Recombinant mouse:
TNF sRI
TNF sRII

Some cross-reactivity was observed with the following:

Factor	Concentration tested (pg/mL)	Observed Value (pg/mL)	% Cross-reactivity
rmTNF-α	12,500	320	2.6

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PLATE LAYOUT

Use this plate layout to record standards and samples assayed.