Quantikine® ELISA

Rat TNF-α Immunoassay

Catalog Number RTA00
SRTA00
PRTA00

For the quantitative determination of rat Tumor Necrosis Factor alpha (TNF-α) concentrations in cell culture supernates, serum, and plasma.

Note: The standard reconstitution method has changed. Please read this package insert in its entirety before using this product.

This package insert must be read in its entirety before using this product.
For research use only. Not for use in diagnostic procedures.

TABLE OF CONTENTS

SECTION	PAGE
INTRODUCTION	1
PRINCIPLE OF THE ASSAY	2
LIMITATIONS OF THE PROCEDURE	2
TECHNICAL HINTS	2
MATERIALS PROVIDED & STORAGE CONDITIONS	3
OTHER SUPPLIES REQUIRED	4
PRECAUTIONS	4
SAMPLE COLLECTION & STORAGE	4
SAMPLE PREPARATION	4
REAGENT PREPARATION	5
ASSAY PROCEDURE	6
CALCULATION OF RESULTS	7
TYPICAL DATA	7
PRECISION	8
RECOVERY	8
LINEARITY	8
SENSITIVITY	9
CALIBRATION	9
SAMPLE VALUES	9
SPECIFICITY	9
REFERENCES	10

MANUFACTURED AND DISTRIBUTED BY:

USA & Canada | R&D Systems, Inc.
614 McKinley Place NE, Minneapolis, MN 55413, USA
TEL: (800) 343-7475 (612) 379-2956 FAX: (612) 656-4400
E-MAIL: info@RnDSystems.com

DISTRIBUTED BY:

UK & Europe | R&D Systems Europe, Ltd.
19 Barton Lane, Abingdon Science Park, Abingdon OX14 3NB, UK
TEL: +44 (0)1235 529449 FAX: +44 (0)1235 533420
E-MAIL: info@RnDSystems.co.uk

China | R&D Systems China Co., Ltd.
24A1 Hua Min Empire Plaza, 726 West Yan An Road, Shanghai PRC 200050
TEL: +86 (21) 52380373 FAX: +86 (21) 52371001
E-MAIL: info@RnDSystemsChina.com.cn

INTRODUCTION

Tumor necrosis factor alpha (TNF-α), also known as cachectin and TNFSF1A, is the prototypic ligand of the TNF superfamily (1). It is a pleiotropic molecule that plays a central role in inflammation, immune system development, apoptosis, and lipid metabolism (2-5). TNF-α is also involved in a number of pathological conditions including asthma, Crohn's disease, rheumatoid arthritis, neuropathic pain, obesity, type 2 diabetes, septic shock, autoimmunity, and cancer (5-11).

Rat TNF-α is synthesized as a 26 kDa type II transmembrane protein that consists of a 35 amino acid (aa) cytoplasmic domain, a 21 aa transmembrane segment, and a 156 aa extracellular domain (ECD) (12). Within the ECD, rat TNF-α shares 95% aa sequence identity with mouse, and 73% - 79% aa identity with bovine, canine, cotton rat, equine, feline, human, rhesus macaque, and porcine TNF-α. It is produced by a wide variety of immune, epithelial, endothelial, and tumor cells. TNF-α is assembled intracellularly to form a noncovalently linked homotrimer which is expressed on the cell surface (13). Cell surface TNF-α can both induce the lysis of tumor cells and virus infected cells, and generate its own downstream cell signaling following ligation by soluble TNF RI (14, 15). Shedding of membrane bound TNF-α by TACE/ADAM17 releases the bioactive cytokine, a 55 kDa soluble trimer of the TNF-α extracellular domain (16-18).

TNF-α binds the ubiquitous 55 - 60 kDa TNF RI (19, 20) and the hematopoietic cell-restricted 78-80 kDa TNF RII (21, 22), both of which are also expressed as homotrimers (1, 23). Both type I and type II receptors bind TNF-α with comparable affinity and can promote NFκB activation (24-27). Only TNF RI, however, contains a cytoplasmic death domain which triggers the activation of apoptosis (3, 28). Soluble forms of both types of receptors are released into human serum and urine and can neutralize the biological activity of TNF-α (29-31).

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 Quantikine kit standards. These results indicate that this 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, Control, and samples are pipetted into the wells and any 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 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 standard diluent, operator, pipetting technique, washing technique, incubation time or temperature, and kit age can cause variation in binding.
- Variations in sample collection, processing, and storage may cause sample value differences.
- This assay is designed to eliminate interference by other factors present in biological samples. Until all factors have been tested in the Quantikine Immunoassay, the possibility of interference cannot be excluded.

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.
- 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.
- 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.

MATERIALS PROVIDED & STORAGE CONDITIONS

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

<table>
<tr><th>PART</th><th>PART #</th><th>CATALOG # RTA00</th><th>CATALOG # SRTA00</th><th>DESCRIPTION</th><th>STORAGE OF OPENED/ RECONSTITUTED MATERIAL</th></tr>
<tr><td>Rat TNF-α Microplate</td><td>890682</td><td>2 plates</td><td>6 plates</td><td>96 well polystyrene microplates (12 strips of 8 wells) coated with a monoclonal antibody specific for rat TNF-α.</td><td>Return unused wells to the foil pouch containing the desiccant pack. Reseal along entire edge of the zip-seal. May be stored for up to 1 month at 2-8 °C.*</td></tr>
<tr><td>Rat TNF-α Standard</td><td>890684</td><td>3 vials</td><td>9 vials</td><td>Recombinant rat TNF-α in a buffered protein base with preservatives; lyophilized. Refer to the vial label for reconstitution volume.</td><td rowspan="2">Use a new Standard and Control for each assay.</td></tr>
<tr><td>Rat TNF-α Control</td><td>890685</td><td>3 vials</td><td>9 vials</td><td>Recombinant rat TNF-α in a buffered protein base with preservatives; lyophilized. The assayed value of the Control should be within the range specified on the label.</td></tr>
<tr><td>Rat TNF-α Conjugate</td><td>892668</td><td>1 vial</td><td>3 vials</td><td>23 mL/vial of a polyclonal antibody specific for rat TNF-α conjugated to horseradish peroxidase with preservatives.</td><td rowspan="7">May be stored for up to 1 month at 2-8 °C.*</td></tr>
<tr><td>Assay Diluent RD1-41</td><td>895514</td><td>1 vial</td><td>3 vials</td><td>12 mL/vial of a buffered protein base with preservatives.</td></tr>
<tr><td>Calibrator Diluent RD5-17</td><td>895512</td><td>2 vials</td><td>6 vials</td><td>21 mL/vial of a buffered protein base with preservatives.</td></tr>
<tr><td>Wash Buffer Concentrate</td><td>895003</td><td>2 vials</td><td>6 vials</td><td>21 mL/vial of a 25-fold concentrated solution of buffered surfactant with preservative. May turn yellow over time.</td></tr>
<tr><td>Color Reagent A</td><td>895000</td><td>1 vial</td><td>3 vials</td><td>12 mL/vial of stabilized hydrogen peroxide.</td></tr>
<tr><td>Color Reagent B</td><td>895001</td><td>1 vial</td><td>3 vials</td><td>12 mL/vial of stabilized chromogen (tetramethylbenzidine).</td></tr>
<tr><td>Stop Solution</td><td>895174</td><td>1 vial</td><td>3 vials</td><td>23 mL/vial of a diluted hydrochloric acid solution.</td></tr>
<tr><td>Plate Sealers</td><td>N/A</td><td>8 strips</td><td>24 strips</td><td colspan="2">Adhesive strips.</td></tr>
</table>

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

RTA00 contains sufficient materials to run ELISAs on two 96 well plates.
SRTA00 (SixPak) contains sufficient materials to run ELISAs on six 96 well plates.

This kit is also available in a PharmPak (R&D Systems, Catalog # PRTA00). PharmPaks contain sufficient materials to run ELISAs on 50 microplates. Specific vial counts of each component may vary. Please refer to the literature accompanying your order for specific vial counts.

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.
- **Polypropylene** test tubes for dilution of standards and samples.

PRECAUTIONS

The Stop Solution provided with this kit is an acid solution.

Some components in this kit contain a preservative which may cause an allergic skin reaction. Avoid breathing mist.

Color Reagent B 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.

SAMPLE COLLECTION & STORAGE

The sample collection and storage conditions listed below are intended as general guidelines. Sample stability has not been evaluated.

Cell Culture Supernates - Remove particulates by centrifugation. 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 before centrifuging for 20 minutes at 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 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 use with this assay.*

SAMPLE PREPARATION

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 of sample + 75 µL of Calibrator Diluent RD5-17.

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 of sample + 100 µL of Calibrator Diluent RD5-17.

REAGENT PREPARATION

Bring all reagents to room temperature before use.

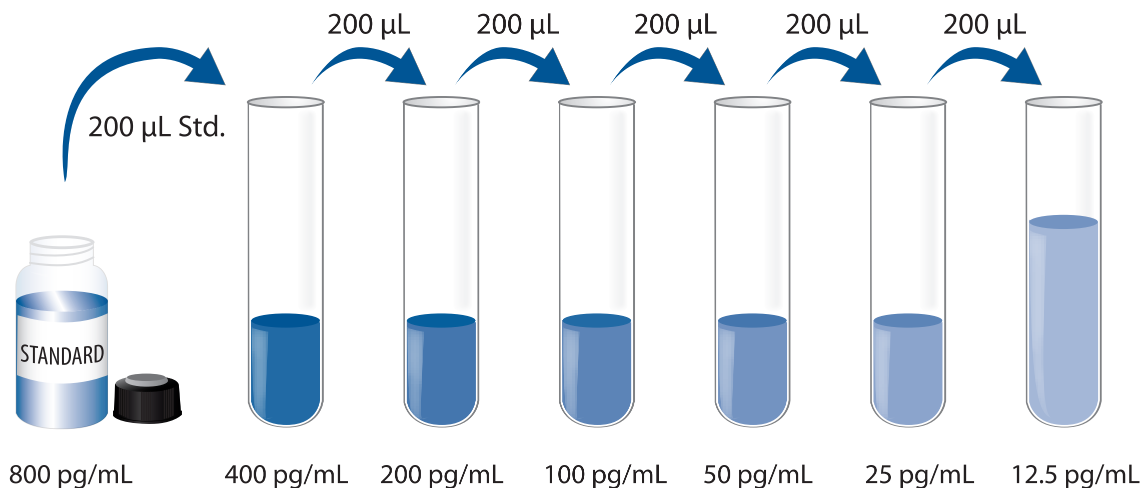
Rat TNF-α Control - Reconstitute the Control with 1.0 mL of deionized or distilled water. Assay the Control undiluted.

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 20 mL of Wash Buffer Concentrate to 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. 100 μL of the resultant mixture is required per well.

Rat TNF-α Standard - **Refer to the vial label for reconstitution volume.** Reconstitute the Rat TNF-α Standard with 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 (800 pg/mL) serves as the high standard. 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 standards, samples, and control 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, and reseal.
3. Add 50 µL of Assay Diluent RD1-41 to each well.
4. Add 50 µL of Standard, Control, or sample* to 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.
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.

CALCULATION OF RESULTS

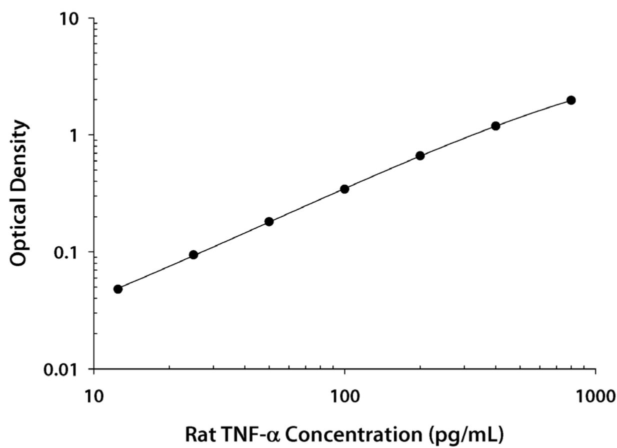
Average the duplicate readings for each standard, control, and sample and subtract the average zero standard optical density (O.D.).

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 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.

Since 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 separate assays to assess inter-assay precision. Assays were performed by at least three technicians using two lots of components.

	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 in samples throughout the range of the assay was evaluated. Samples were diluted prior to assay.

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

LINEARITY

To assess the linearity of the assay, samples spiked with various concentrations of rat TNF-α were diluted with Calibrator Diluent and then assayed. Samples were diluted prior to assay.

		Cell culture supernates (n=4)	Serum (n=4)	EDTA plasma (n=4)	Heparin plasma (n=4)
1:2	Average % of Expected	94	100	100	106
	Range (%)	93-94	96-103	97-103	102-108
1:4	Average % of Expected	95	99	96	108
	Range (%)	91-97	96-105	93-100	106-112
1:8	Average % of Expected	96	95	92	105
	Range (%)	94-96	92-101	88-98	103-107
1:16	Average % of Expected	94	94	96	108
	Range (%)	89-97	90-100	93-104	104-114

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SENSITIVITY

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

The MDD 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.

SAMPLE VALUES

Serum/Plasma - Forty serum samples and thirteen 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 cell culture supernate was removed, assayed for levels of rat TNF-α and measured 900 pg/mL.

SPECIFICITY

This assay recognizes natural and recombinant rat TNF-α.

The factors listed below were prepared at 50 ng/mL in Calibrator Diluent 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 rat:

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

Recombinant mouse:

TNF RI
TNF RII

Recombinant human:

TNF-α
TNF-β
TNF RI
TNF RII

Recombinant mouse TNF-α cross-reacts approximately 2.6% in this assay.

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