Quantikine® ELISA

Mouse/Rat TNF RI/TNFRSF1A Immunoassay

Catalog Number MRT10

For the quantitative determination of mouse or rat Tumor Necrosis Factor Receptor I (TNF RI) concentrations in cell culture supernates and serum.

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

Mouse TNF RI is the prototypical member of the TNF receptor superfamily that includes molecules as diverse as NGFR/p75, CD40, CD27, Fas, and TRAIL receptors(1). TNF RI is a 55-60 kDa, 425 amino acid (aa) type I transmembrane glycoprotein that contains a 219 aa cytoplasmic segment, a 23 aa transmembrane domain, and a 183 aa extracellular region rich in cysteine residues (2). The cytoplasmic segment contains a superfamily-typical "death domain" that interacts with cytoplasmic TRADD and initiates cellular apoptosis (1). The extracellular domain of mouse TNF RI shares 70% and 89% aa identity with human and rat TNF RI, respectively (2-5). Cells known to express TNF RI include fibroblasts (6), B cells (7), keratinocytes (8), microglia, astrocytes, and oligodendroglia (9), $CD34^+$ stem cells (10), monocytes (11), dendritic cells (8, 12), cardiac muscle cells (13), megakaryocytes (14), Schwann cells (15), neutrophils (16), adipocytes (17), endothelial cells (18), NK cells (19), and macrophages (20).

TNF RI has been shown to bind TNF-α with high affinity (2). In addition, TNF RI has also been shown to bind lymphotoxin-α_3 homotrimer and the lymphotoxin-$\alpha_2\beta_1$ heterotrimer (21). Unlike TNF RII, the expression level of TNF RI appears to be stable and relatively unaffected by cytokines and activating molecules (22). Ligation of TNF to TNF RI can stimulate the production of parasite-protective nitric oxide (23) and induce the internalization of TNF RI and release of soluble TNF RII (24). Both TNF RI and TNF RII are required for induction of TNF-mediated apoptosis of multiple cell types (25, 26).

Soluble TNF RI, generated by proteolytic cleavage of the cell surface receptor (1, 27), has been shown to bind TNF with high-affinity. It has been postulated that soluble TNF receptors may inhibit circulating TNF activity (28). Alternatively, soluble TNF R can also potentiate TNF activity by stabilizing the trimeric structure of physiologic TNF-α (29).

The Quantikine® Mouse/Rat TNF RI/TNFRSF1A Immunoassay is a 4.5 hour solid-phase ELISA designed to measure mouse or rat TNF RI in cell culture supernates and serum. It contains *E. coli*-expressed recombinant mouse TNF RI and antibodies raised against the recombinant factor. This immunoassay has been shown to accurately quantitate the recombinant mouse TNF RI. Results obtained using natural mouse or rat TNF RI 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 mouse and rat TNF RI.

PRINCIPLE OF THE ASSAY

This assay employs the quantitative sandwich enzyme immunoassay technique. A polyclonal antibody specific for mouse/rat TNF RI has been pre-coated onto a microplate. Standards, control, and samples are pipetted into the wells and any TNF RI present is bound by the immobilized antibody. After washing away any unbound substances, an enzyme-linked polyclonal antibody specific for mouse/rat TNF RI 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 RI 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 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.
- 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.

PART	PART #	DESCRIPTION	STORAGE OF OPENED/ RECONSTITUTED MATERIAL
Mouse/Rat TNF RI Microplate	890026	96 well microplate (12 strips of 8 wells) coated with a polyclonal antibody specific for mouse/rat TNF RI.	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.*
Mouse/Rat TNF RI Standard	890080	Recombinant mouse TNF RI in a buffered protein base with preservatives; lyophilized. *Refer to the vial label for reconstitution volume.*	Aliquot and store for up to 1 month at ≤ -20 °C in a manual defrost freezer.*
Mouse/Rat TNF RI Control	890024	Recombinant mouse TNF RI in a buffered protein base with preservatives; lyophilized. The assay value of the control should be within the range specified on the label.	
Mouse/Rat TNF RI Conjugate	890079	12 mL of a polyclonal antibody specific for mouse/rat TNF RI conjugated to horseradish peroxidase with preservatives.	May be stored for up to 1 month at 2-8 °C.*
Assay Diluent RD1W	895038	12 mL of a buffered protein base with preservatives.	
Calibrator Diluent RD5Y	895201	21 mL of a buffered protein base with preservatives.	
Wash Buffer Concentrate	895003	21 mL of a 25-fold concentrated solution of buffered surfactant with preservative. *May turn yellow over time.*	
Color Reagent A	895000	12 mL of stabilized hydrogen peroxide.	
Color Reagent B	895001	12 mL of stabilized chromogen (tetramethylbenzidine).	
Stop Solution	895174	23 mL of diluted hydrochloric acid.	
Plate Sealers	N/A	4 adhesive strips.	

* 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.
- 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. 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 2000 x g. Remove serum and assay immediately or aliquot and store samples at ≤ -20 °C. Avoid repeated freeze-thaw cycles.

Note: *Grossly hemolyzed or lipemic samples are not suitable for use in this assay.*

SAMPLE PREPARATION

Mouse serum samples require a 10-fold dilution into Calibrator Diluent RD5Y. A suggested 10-fold dilution is 20 µL of sample + 180 µL of Calibrator Diluent RD5Y.

Rat serum samples require a 4-fold dilution into Calibrator Diluent RD5Y. A suggested 4-fold dilution is 50 µL of sample + 150 µL of Calibrator Diluent RD5Y.

REAGENT PREPARATION

Bring all reagents to room temperature before use.

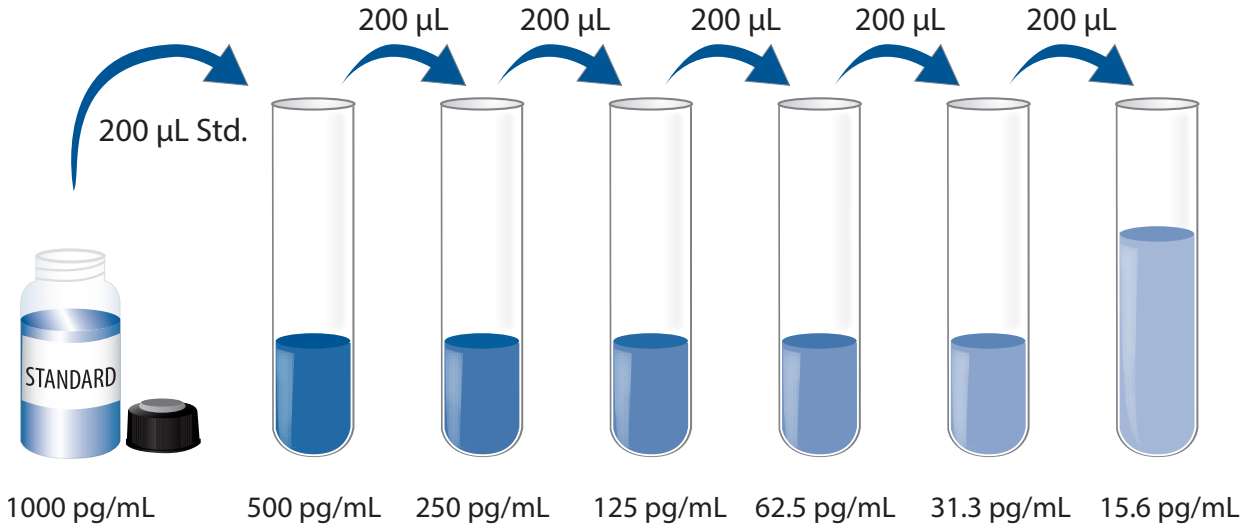
Mouse/Rat TNF RI Control - Reconstitute the control with 1.0 mL of deionized or distilled water. Mix thoroughly. Assay the control undiluted.

Wash Buffer - If crystals have formed in the concentrate, warm to room temperature and mix gently until the crystals have completely dissolved. 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.

Mouse/Rat TNF RI Standard - **Refer to the vial label for reconstitution volume.** Reconstitute the Mouse/Rat TNF RI Standard with Calibrator Diluent RD5Y. Do not substitute other diluents. This reconstitution produces a stock solution of 1000 pg/mL. Allow the stock solution to sit for a minimum of 5 minutes with gentle mixing prior to making dilutions.

Pipette 200 µL of Calibrator Diluent RD5Y into each tube. Use the stock solution to produce a dilution series (below). Mix each tube thoroughly before the next transfer. The undiluted Mouse/Rat TNF RI Standard (1000 pg/mL) serves as the high standard. Calibrator Diluent RD5Y 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, control, and samples be assayed in duplicate.

1. Prepare all 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 RD1W to each well.
4. Add 50 µL of standard, control, or sample* per 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 decanting. Invert the plate and blot it against clean paper towels.
6. Add 100 µL of Mouse/Rat TNF RI 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 may require dilution. See Sample Preparation section.

CALCULATION OF RESULTS

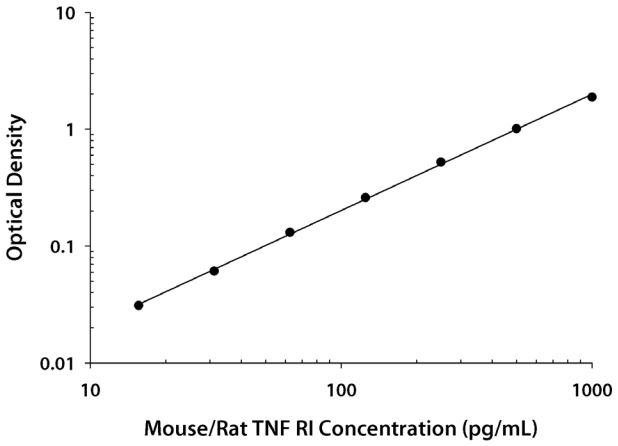
Average the duplicate readings for each standard, control, and sample and subtract the average zero standard optical density (O.D.).

Create a standard curve by reducing the data using computer software capable of generating a log/log 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 a log/log graph. The data may be linearized by plotting the log of the mouse/rat TNF RI concentrations versus the log of the O.D. on a linear scale, and the best fit line can be determined by regression analysis.

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	0.024 0.025	0.024	—
15.6	0.055 0.055	0.055	0.031
31.3	0.084 0.087	0.085	0.061
62.5	0.155 0.155	0.155	0.131
125	0.279 0.290	0.284	0.260
250	0.533 0.562	0.548	0.524
500	1.005 1.065	1.035	1.011
1000	1.828 1.972	1.900	1.876

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)	46.0	118	581	48.3	120	592
Standard deviation	2.7	6.5	27.5	2.5	6.0	33.3
CV (%)	5.9	5.5	4.7	5.2	5.0	5.6

RECOVERY

The recovery of mouse/rat TNF RI spiked to levels throughout the range of the assay in various matrices was evaluated.

Sample Type	Average % Recovery	Range
Mouse cell culture supernates (n = 5)	97	89-109%
Mouse serum* (n = 5)	96	83-104%
Rat cell culture supernates (n=5)	98	92-116%
Rat serum* (n=5)	96	88-104%

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

LINEARITY

To assess the linearity of the assay, samples containing and/or spiked with high concentrations of mouse/rat TNF RI in each matrix were diluted with calibrator diluent and assayed.

		Cell culture supernates (n=4)	Mouse serum* (n=4)	Rat serum* (n=4)
1:2	Average % of Expected	99	101	103
	Range (%)	92-104	97-104	99-106
1:4	Average % of Expected	102	101	106
	Range (%)	96-109	95-105	104-111
1:8	Average % of Expected	100	102	106
	Range (%)	93-105	95-113	99-108
1:16	Average % of Expected	99	99	103
	Range (%)	93-108	89-113	96-106

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

SENSITIVITY

Twenty-two assays were evaluated and the minimum detectable dose (MDD) of mouse/rat TNF RI ranged from 0.5-2.4 pg/mL. The mean MDD was 1.1 pg/mL.

The MDD was determined by adding two standard deviations to the mean O.D. value of twenty zero standard replicates and calculating the corresponding concentration.

CALIBRATION

This immunoassay is calibrated against a highly purified *E. coli*-expressed recombinant mouse TNF RI produced at R&D Systems®.

SAMPLE VALUES

Serum/Plasma - Samples were evaluated for the presence of mouse and rat TNF RI in this assay.

	Mean (pg/mL)	Range (pg/mL)	Standard Deviation (pg/mL)
Mouse serum (n=40)	891	575-1575	249
Rat serum (n=20)	332	218-980	162

Cell Culture Supernates - L-929 mouse fibroblast cells (1 x 10^5 cells/mL) were cultured in MEM containing L-glutamine and 10% equine serum for 3 days. The cell culture supernate was removed, assayed for mouse TNF RI, and measured 461 pg/mL.

SPECIFICITY

This assay recognizes natural and recombinant mouse and rat TNF RI.

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 mouse/rat TNF RI control were assayed for interference. No significant cross-reactivity or interference was observed.

Recombinant mouse:

C10
Eotaxin
G-CSF
GM-CSF
IFN-γ
IL-1α
IL-1β
IL-1ra
IL-2
IL-3
IL-4
IL-5
IL-6
IL-7
IL-9
IL-10
IL-10 R
IL-12
IL-13
IL-17
IL-18
JE/MCP-1
KC
Leptin
LIF
M-CSF
MIP-1α
MIP-1β
MIP-2
OSM
SCF
TNF-α
TNF-β
TNF RII
Tpo
VEGF

Recombinant human:

TNF-α
TNF-β
TNF RI
TNF RII

Other recombinants:

rat TNF-α
canine TNF RI

REFERENCES

1. Lotz, M. *et al.* (1996) J. Leukoc. Biol. **60**:1.
2. Lewis, M. *et al.* (1991) Proc. Natl. Acad. Sci. USA **88**:2830.
3. Schall, T.J. *et al.* (1990) Cell **61**:361.
4. Loetscher, H. *et al.* (1990) Cell **61**:351.
5. Himmler, A. *et al.* (1990) DNA Cell Biol. **9**:705.
6. Debets, R. *et al.* (1996) Cytokine **8**:80.
7. Erikstein, B.K. *et al.* (1991) Eur. J. Immunol. **21**:1033.
8. Kristensen, M. *et al.* (1993) Clin. Exp. Immunol. **94**:354.
9. Dopp. J.M. *et al.* (1997) J. Neuroimmunol. **75**:104.
10. Sato, T. *et al.* (1997) Br. J. Haematol. **97**:356.
11. van der Poll, T. *et al.* (1997) J. Immunol. **158**:1490.
12. Yamada, K. *et al.* (1997) Blood **90**:4832.
13. Krown, K.A. *et al.* (1995) FEBS Lett. **376**:24.
14. Tacchini-Cottier, F. *et al.* (1998) J. Immunol. **160**:6182.
15. Skoff, A.M. *et al.* (1998) J. Neurosci. Res. **53**:747.
16. Lantz, M. *et al.* (1994) J. Immunol. **152**:1362.
17. Duo, D. *et al.* (1998) J. Immunol. **160**:2742.
18. Paleolog, E.M. *et al.* (1994) Blood **84**:2578.
19. Naume, B. *et al.* (1991) J. Immunol. **146**:3045.
20. Tannenbaum, C.S. *et al.* (1993) J. Immunol. **151**:6833.
21. von Boehmer, H. (1997) Proc. Natl. Acad. Sci. USA **94**:8926.
22. Vandenabeele, P. *et al.* (1995) Trends Cell Biol. **5**:392.
23. Deckert-Schluter, M. *et al.* (1998) J. Immunol. **160**:3427.
24. Higuchi, M. and B.B. Aggarwal (1994) J. Immunol. **152**:3550.
25. Lucas, R. *et al.* (1998) Eur. J. Immunol. **28**:3577.
26. Vandenabeele, P. *et al.* (1995) J. Immunol. **154**:2904.
27. Nophar, Y. *et al.* (1990) EMBO J. **9**:3269.
28. Terlizzese, M. *et al.* (1996) J. Interferon Cytokine Res. **16**:1047.
29. Aderka, D. *et al.* (1992) J. Exp. Med. **175**:323.