Quantikine® QuicKit™ ELISA

Human TNF RI/TNFRSF1A Immunoassay

Catalog Number QK225

For the quantitative determination of human Tumor Necrosis Factor Receptor I (TNF RI) concentrations in cell culture supernates, serum, plasma, and urine.

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 factors (TNFs) are pleiotropic cytokines that are considered primary modifiers of the inflammatory and immune reactions of animals produced in response to injury or infection. Two forms of TNF, designated TNF-α (or cachectin) and TNF-β (or lymphotoxin), have been described that share 30% sequence similarity and compete for binding to the same receptors. TNFs play a necessary and beneficial role as mediators of host resistance to infections and tumor formation. However, over-production or inappropriate expression of these factors can lead to a variety of pathological conditions, including wasting, systemic toxicity, and septic shock. For reviews of the literature relating to these factors, see references 1 and 2.

The actions of TNFs are produced subsequent to binding of the factors to cell surface receptors. Two distinct TNF receptors have been identified and cloned. Virtually all cell types studied show the presence of one or both of these receptor types. One receptor type, termed TNF RII (Type A, Type a, 75 kDa or utr antigen), shows an apparent molecular weight of 75 kDa. The gene for this receptor encodes a presumptive transmembrane protein of 439 amino acid (aa) residues (3, 19). The other receptor type, termed TNF RI (Type B, Type b, 55 kDa or htr antigen), shows an apparent molecular weight of 55 kDa. The gene for this protein encodes a transmembrane protein of 426 aa residues (4, 5, 19). Both receptor types show high affinity binding of either TNF-α or TNF-β. The two receptor types are immunologically distinct but their extracellular domains show similarities in the pattern of cysteine residue locations in four domains (3). The intracellular domains of the two receptor types are apparently unrelated, suggesting the possibility that the two receptor types employ different signal transduction pathways.

Several groups have identified soluble TNF binding proteins in human serum and urine (6-8) that can neutralize the biological activities of TNF-α and TNF-β. Two types have been identified and designated sTNF RI (or TNF BPI) and sTNF RII (or TNF BPII). These soluble forms have now been shown to represent truncated forms of the two types of TNF receptors discussed above. The soluble receptor forms apparently arise as a result of shedding of the extracellular domains of the receptors, and concentrations of about 1-2 ng/mL are found in the serum and urine of healthy subjects (9, 10). The levels of the soluble receptors vary from individual to individual but are stable over time for given individuals (9).

Elevated levels of TNF receptors have been found in the amniotic fluid and urine of pregnant women (11), in serum or plasma in association with pathological conditions such as endotoxinemia (12, 13), meningiococcemia (14), and HIV infection (15), and in plasma and ascites of patients in association with infections and malignancies (16). The mechanisms involved in the induction of shedding of the TNF receptors are not well understood. There are reports of correlations between increased TNF levels and soluble receptor levels, suggesting generally that stimuli that cause TNF levels to rise also induce shedding of TNF receptors (12-14, 17). There is also evidence, however, that suggests the shedding of the two types of soluble receptors is independently regulated (13).

The physiological role of the soluble TNF receptors is not known. It is known that both types of soluble receptors can bind to TNF *in vitro* and inhibit its biological activity by competing with cell surface receptors for TNF binding. Consequently it has been suggested that shedding of soluble receptors in response to TNF release could serve as a mechanism for binding and inhibiting the TNF not immediately bound to surface receptors, thus protecting other cells from the effects of TNF and localizing the inflammatory response (12, 17). It is also possible that shedding of receptors represents a mechanism for desensitizing the cells that shed the receptors from the effects of TNF (17). On the other hand, it has been reported that at low concentrations of TNF, binding to soluble receptors can stabilize TNF and augment some of its activities (18). Thus it is possible that under some conditions the pool of TNF bound to soluble receptors could represent a reservoir for the stabilization and controlled release of TNF.

The Quantikine® QuicKit™ Human TNF RI/TNFRSF1A Immunoassay is a one step, 80-minute solid phase ELISA designed to measure human TNF RI levels in cell culture supernates, serum, plasma, and urine. It contains *E. coli*-expressed recombinant human TNF RI and antibodies raised against the recombinant protein. Results obtained for natural human TNF RI showed linear curves that were parallel to the standard curves obtained using the recombinant QuicKit™ standards. These results indicate that this kit can be used to determine relative mass values for natural human TNF RI.

PRINCIPLE OF THE ASSAY

This assay employs the quantitative sandwich enzyme immunoassay technique. An anti-tag antibody has been pre-coated onto a microplate. Standards and samples are pipetted into the wells followed by an antibody cocktail. The antibody cocktail consists of an affinity tag labeled monoclonal capture antibody and an enzyme-linked polyclonal detection antibody, specific for human TNF RI. After washing away any unbound substances, a substrate solution is added to the wells and color develops in proportion to the amount of TNF RI bound. 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 those from other lots or sources.
- If samples generate values higher than the highest standard, further dilute the samples with the 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® QuicKit™ 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.
- Ensure reagent addition to plate wells is uninterrupted.
- To ensure accurate results, proper adhesion of plate sealers during incubation steps is necessary.
- 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.
- 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.

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
QuicKit™ Coated Microplate	899063	96 well polystyrene microplate (12 strips of 8 wells) coated with an anti-tag monoclonal antibody.	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.*
Human TNF RI Standard	899088	2 vials of recombinant human TNF RI in a buffered protein solution with preservatives; lyophilized. *Refer to the vial label for reconstitution volume.*	Use a new standard for each assay. Discard after use.
Human TNF RI Capture Ab Concentrate	899086	Lyophilized tagged monoclonal antibody specific for human TNF RI.	May be stored for up to 1 month at 2-8 °C.*
Human TNF RI Detection Ab Concentrate	899087	400 µL of a polyclonal antibody specific for human TNF RI conjugated to horseradish peroxidase with preservatives.	
Calibrator Diluent RD5-74	896167	2 vials of 21 mL/vial 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	895032	6 mL of 2 N sulfuric acid.	
Plate Sealers	N/A	4 adhesive strips.	

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

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 SDS on our website prior to use.

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.
- Horizontal orbital microplate shaker (0.12" orbit) capable of maintaining a speed of 500 ± 50 rpm.
- Test tubes for dilution of standards and samples.
- Human TNF RI Controls (optional; R&D Systems®, Catalog # QC270).

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 and assay immediately or aliquot and store samples at ≤ -20 °C. Avoid repeated freeze-thaw cycles.

Serum - Use a serum separator tube (SST) and allow samples to clot for 30 minutes at room temperature before centrifugation for 15 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 15 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: *Citrate plasma has not been validated for use in this assay.*

Urine - Aseptically collect the first urine of the day (mid-stream), voided directly into a sterile container. Centrifuge to remove particulate matter, assay immediately or aliquot and store at ≤ -20 °C. Avoid repeated freeze-thaw cycles.

SAMPLE PREPARATION

Serum, plasma, and urine samples require at least a 2-fold dilution due to a matrix effect. A 4-fold dilution is recommended due to high endogenous levels. A suggested 4-fold dilution is 50 µL of sample + 150 µL of Calibrator Diluent RD5-74.

REAGENT PREPARATION

Bring all reagents to room temperature before use.

Human TNF RI Capture Ab Concentrate - **Refer to the vial label for reconstitution volume.** Reconstitute the Human TNF RI Capture Ab Concentrate with Calibrator Diluent RD5-74. This reconstitution produces a 20X Capture Antibody stock. Allow the capture antibody to sit for a minimum of 5 minutes with gentle agitation prior to diluting. Once reconstituted, the 20X Capture Antibody stock can be stored for 4 weeks at 2-8 °C.

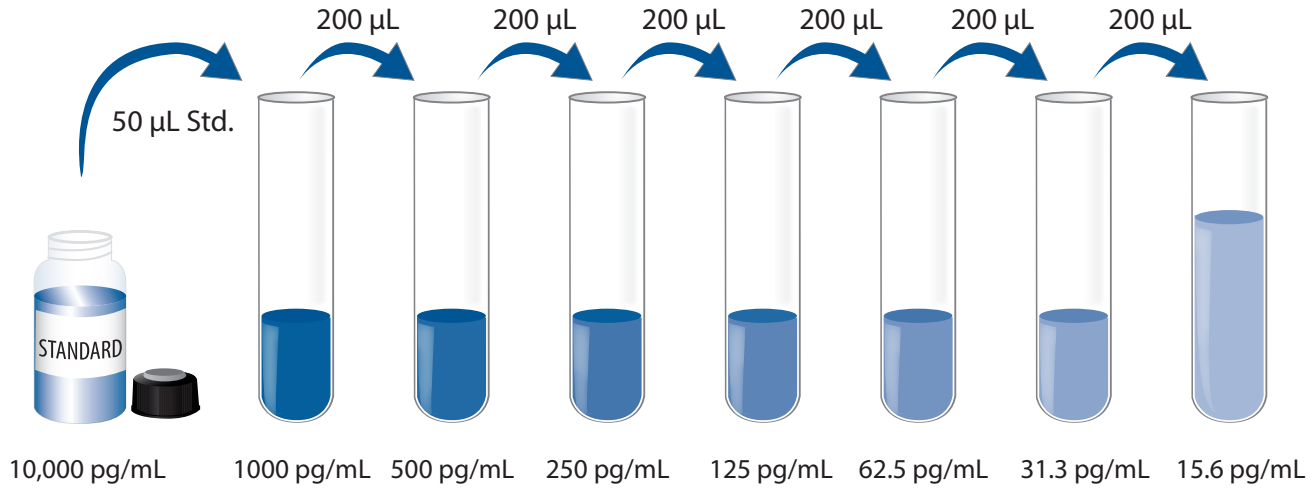
Antibody Cocktail - Dilute the reconstituted Capture Ab stock and the Detection Ab Concentrate 20-fold in Calibrator Diluent RD5-74. For a full plate, add 300 µL of reconstituted Human TNF RI Capture Ab stock and 300 µL of Human TNF RI Detection Ab Concentrate to 5.4 mL of Calibrator Diluent RD5-74.

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 480 mL of 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.

Human TNF RI Standard - **Refer to the vial label for reconstitution volume.** Reconstitute the Human TNF RI Standard with deionized or distilled water. 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 450 µL of Calibrator Diluent RD5-74 into the 1000 pg/mL tube. Pipette 200 µL into each remaining tube. Use the stock solution to produce a dilution series (below). Mix each tube thoroughly before the next transfer. The 1000 pg/mL standard serves as the high standard. The Calibrator Diluent RD5-74 serves as the zero standard (0 pg/mL).



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ASSAY PROCEDURE

Bring all reagents and samples to room temperature before use. It is recommended that all standards, controls, and samples 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, and reseal.
3. Add 50 µL of standard, control, or sample* per well.
4. Add 50 µL Antibody Cocktail to each well. Cover with the adhesive strip provided. Incubate for 1 hour at room temperature on a horizontal orbital microplate shaker (0.12″ orbit) set at 500 ± 50 rpm.
5. Aspirate each well and wash, repeating the process twice for a total of three 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 Substrate Solution to each well. Incubate for 20 minutes at room temperature **on the benchtop. Protect from light.**
7. 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.
8. 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

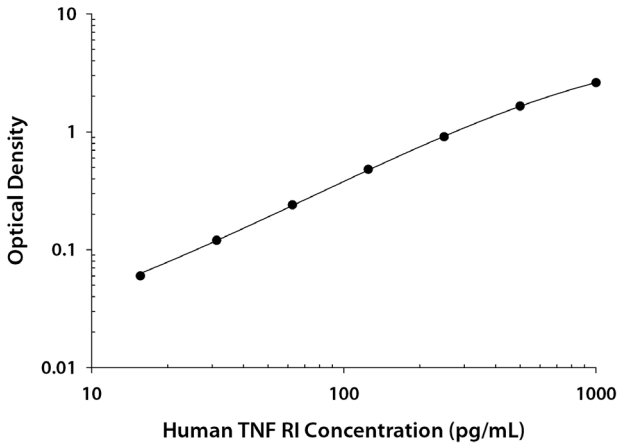
Average the duplicate readings for each standard, control, and sample and subtract the average zero standard optical density (O.D.).

Create a standard curve by reducing the data using computer software capable of generating a 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 human TNF RI 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.

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	0.023		——
	0.026	0.025	
15.6	0.082		
	0.087	0.085	0.060
31.3	0.143		
	0.146	0.145	0.120
62.5	0.264		
	0.266	0.265	0.240
125	0.495		
	0.496	0.496	0.471
250	0.931		
	0.934	0.933	0.908
500	1.672		
	1.683	1.678	1.653
1000	2.616		
	2.670	2.643	2.618

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PRECISION

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

Inter-Assay Precision (Precision between assays)
Two samples of known concentration were tested in ten separate assays to assess inter-assay precision. Assays were performed by at least three technicians.

	Intra-Assay Precision		Inter-Assay Precision	
Sample	**1**	**2**	**1**	**2**
n	20	20	10	10
Mean (pg/mL)	100	624	99.9	619
Standard deviation	1.75	17.0	6.78	39.3
CV (%)	1.8	2.7	6.8	6.3

RECOVERY

The recovery of human TNF RI spiked to three different levels in samples throughout the range of the assay in various matrices was evaluated.

Sample Type	Average % Recovery	Range
Cell culture media (n=4)	101	92-109%
Serum* (n=2)	99	91-113%
EDTA plasma* (n=2)	98	88-108%
Heparin plasma* (n=2)	98	85-109%
Urine* (n=2)	96	92-100%

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

SENSITIVITY

Fourteen assays were evaluated and the minimum detectable dose (MDD) of human TNF RI ranged from 0.420-1.69 pg/mL. The mean MDD was 0.894 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.

LINEARITY

To assess the linearity of the assay, samples containing and/or spiked with high concentrations of human TNF RI were diluted with calibrator diluent to produce samples with values within the dynamic range of the assay.

		Cell culture media (n=4)	Serum* (n=2)	EDTA plasma* (n=2)	Heparin plasma* (n=2)	Urine* (n=4)
1:2	Average % of Expected	102	95	96	94	98
	Range (%)	101-103	91-99	95-98	92-96	95-101
1:4	Average % of Expected	102	95	97	95	99
	Range (%)	101-104	92-99	95-98	94-97	97-101
1:8	Average % of Expected	103	98	100	99	100
	Range (%)	100-105	94-101	100-101	98-100	97-103
1:16	Average % of Expected	106	101	102	102	101
	Range (%)	101-111	97-105	100-103	100-105	98-106

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

CALIBRATION

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

SAMPLE VALUES

Serum/Plasma/Urine - Samples from apparently healthy volunteers were evaluated for the presence of human TNF RI in this assay. No medical histories were available for the donors used in this study.

Sample Type	Mean (pg/mL)	Range (pg/mL)	Standard Deviation (pg/mL)
Serum (n=10)	1341	1050-1948	257
EDTA plasma (n=10)	1092	894-1636	233
Heparin plasma (n=10)	1102	861-1600	251
Urine (n=10)	1408	836-2140	485

Cell Culture Supernates - Human peripheral blood mononuclear cells (PBMCs) (1×10^6 cells/mL) were cultured in RPMI 1640 supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 100 U/mL penicillin, and 100 µg/mL streptomycin sulfate. Cells were left unstimulated, stimulated with 10 µg/mL PHA, or 5 ug/mL Concanavalin A for 5 days. Aliquots of the cell culture supernates were removed and assayed for levels of human TNF RI.

Condition	Day 5 (pg/mL)
Unstimulated	172
Stimulated with PHA	280
Stimulated with Concanavalin A	234

SPECIFICITY

This assay recognizes natural and recombinant human 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 at 50 ng/mL in a low-level range recombinant human TNF RI control were assayed for interference. No significant cross-reactivity or interference was observed.

Recombinant human:

FADD
TNF-β
TNF RII
TRADD

Recombinant mouse:

TNF-β
TNF RI

Recombinant canine TNF RI cross-reacts approximately 11.5 % and interferes at concentrations > 125 pg/mL in this assay.

Recombinant human and rat TNF-α do not cross-react but do interfere at concentrations > 2 ng/mL in this assay.

Recombinant mouse and porcine TNF-α do not cross-react but do interfere at concentrations > 5 ng/mL in this assay.

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