

Quantikine[®]

Human TNF- α Immunoassay

Catalog Number DTA00C

STA00C

PDTA00C

For the quantitative determination of human tumor necrosis factor alpha (TNF- α) concentrations in cell culture supernates, 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

The prototype ligand of the TNF superfamily, TNF- α , is a pleiotropic cytokine that plays a central role in inflammation and apoptosis (1 - 4). It is synthesized as a 26 kDa, type II transmembrane protein that is 233 amino acids in length (4, 5). It contains a 30 amino acid (aa) cytoplasmic domain, a 26 aa transmembrane segment, and a 177 aa extracellular region (6, 7). TNF- α is assembled intracellularly to form a transmembrane, non-covalently-linked homotrimeric protein. The 157 aa residue soluble form of TNF- α (sTNF- α) is released from the C-terminus of the transmembrane protein through the activity of TNF- α -converting enzyme (TACE), a membrane-bound disintegrin metalloproteinase (8, 9). Human cells known to express TNF- α include B cells (10), colonic columnar epithelial cells (11), NK and CD3⁺CD56⁺ hepatic natural T cells (12), macrophages (13), monocytes and monocyte-derived dendritic cells (14), CD4⁺ and CD8⁺ T cells (15), mast cells (16), neutrophils (17), keratinocytes (18), plasma cells (19), and adipocytes (20).

The two identified high affinity receptors for human TNF- α are TNF RI/TNFRSF1A and TNF RII/TNFRSF1B. While each possess a typical TNF R structure, these receptors have relatively little aa identity and exhibit differences in receptor/ligand kinetics and cellular signaling. The dissociation rate of sTNF- α from TNF RII is significantly faster than that of TNF RI (21), and while the membrane (mTNF- α) and soluble forms of TNF- α activate TNF RI equally well, TNF RII is activated more effectively by mTNF- α (22). TNF RI is a 55 - 60 kDa, 415 aa, type I transmembrane glycoprotein (23 - 25). The cytoplasmic domain is notable for the presence of an 80 aa "death domain" motif known for its capacity to mediate apoptosis (26). TNF- α activation of TNF RI, and internalization of the ligand/receptor complex, leads to the recruitment of adaptor proteins including TNF receptor-associated death domain (TRADD) (27) and Fas-associated death domain (FADD) (28), ultimately leading to the activation of downstream effectors and apoptosis. In addition, TRADD interaction with TNF receptor-associating factors (TRAFs) can initiate anti-apoptotic NF- κ B signaling pathways (28 - 30). TNF RII is a 75 - 80 kDa, transmembrane glycoprotein that lacks the cytoplasmic death domain (31, 32). However, the receptor retains the ability to recruit TRAF2, leading to downstream NF- κ B signaling (33), and in some cells, TNF RII-mediated apoptotic pathways that may involve crosstalk between TNF RI and TNF RII receptors (34, 35). Although TNF RI and TNF RII can individually mediate TNF- α functions, some physiological activities apparently require the presence and interaction of both receptors (36 - 39). A novel TNF RII isoform, generated via alternative splicing, results in an intracellularly expressed 50 kDa receptor termed icp75TNFR (40). Over-expression of icp75TNFR results in TRAF2-mediated NF- κ B activation suggesting the potential for intracellular TNF- α activity (40).

The role of TNF- α and/or its receptors in host defense and inflammatory responses has been well documented. TNF- α is reported to promote inflammatory cell infiltration by upregulating leukocyte adhesion molecules on endothelial cells, serve as a chemotactic agent for monocytes, and activate phagocyte killing mechanisms (30). Deficiencies in either TNF- α or its receptors can increase susceptibility to infection by intracellular pathogens (41 - 44). TNF- α may also play a role in lymphoid tissue development. Knockout mice lack splenic B cell follicles and the ability to form germinal centers (45, 46). Other potential physiological roles for TNF- α and its receptors include regulating the differentiation of hematopoietic stem and progenitor cells (47 - 49).

TNF- α has been implicated in a number of pathophysiological processes. It is associated with unregulated pro-inflammatory activity and is thought to be a critical mediator of endotoxin-induced septic shock (50). Cachexia (or whole body wasting) has also been associated with long-term circulating TNF- α (51, 52). Other disorders with potential TNF- α involvement include asthma (53), type 2 diabetes (54), Crohn's disease (55), and rheumatoid arthritis (56).

The Quantikine Human TNF- α Immunoassay is a 3.5 - 4.5 hour solid phase ELISA designed to measure human TNF- α in cell culture supernates, serum, and plasma. It contains *E. coli*-derived recombinant human TNF- α and antibodies raised against this protein. It has been shown to accurately quantitate the recombinant factor. Results obtained with naturally occurring TNF- α samples showed linear curves that were parallel to the standard curves obtained using the Quantikine kit standards. These results indicate that the Quantikine Immunoassay kit can be used to determine relative mass values for natural human TNF- α .

Since the measurement of TNF- α is insensitive to the addition of recombinant forms of either of the two types of soluble receptors, it is probable that this measurement detects the total amount of TNF- α in samples, *i.e.*, the total amount of free TNF- α plus the amount of TNF- α bound to soluble receptors.

PRINCIPLE OF THE ASSAY

This assay employs the quantitative sandwich enzyme immunoassay technique. A monoclonal antibody specific for TNF- α has been pre-coated onto a microplate. Standards 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 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 and color develops in proportion to the amount of TNF- α 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 those from 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 the appropriate 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 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.

MATERIALS PROVIDED

Description	Part #	Cat. # DTA00C	Cat. # STA00C
TNF-α Microplate - 96 well polystyrene microplate (12 strips of 8 wells) coated with a mouse monoclonal antibody against TNF- α .	890123	1 plate	6 plates
TNF-α Conjugate - 21 mL/vial of polyclonal antibody against TNF- α conjugated to horseradish peroxidase with preservatives.	892539	1 vial	6 vials
TNF-α Standard - Recombinant human TNF- α in a buffered protein base with preservatives; lyophilized.	892540	1 vial	6 vials
Assay Diluent RD1F - 6 mL/vial of a buffered protein base with preservatives. Contains a precipitate. Mix well before and during use.	895041	1 vial	6 vials
Calibrator Diluent RD6-35 - 21 mL/vial of animal serum with preservatives.	895360	1 vial	6 vials
Wash Buffer Concentrate - 21 mL/vial of a 25-fold concentrated solution of buffered surfactant with preservatives.	895003	1 vial	6 vials
Color Reagent A - 12.5 mL/vial of stabilized hydrogen peroxide.	895000	1 vial	6 vials
Color Reagent B - 12.5 mL/vial of stabilized chromogen (tetramethylbenzidine).	895001	1 vial	6 vials
Stop Solution - 6 mL/vial of 2 N sulfuric acid.	895032	1 vial	6 vials
Plate Covers - Adhesive strips.	—	4 strips	24 strips

DTA00C contains sufficient materials to run an ELISA on one 96 well plate.

STA00C (SixPak) contains sufficient materials to run ELISAs on six 96 well plates.

This kit is also available in a PharmPak (R&D Systems, Catalog # PDTA00C). 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.

STORAGE

Unopened Kit	Store at 2 - 8° C. Do not use past kit expiration date.	
Opened/ Reconstituted Reagents	Diluted Wash Buffer	May be stored for up to 1 month at 2 - 8° C.*
	Stop Solution	
	Calibrator Diluent RD6-35	
	Assay Diluent RD1F	
	Conjugate	
	Unmixed Color Reagent A	
	Unmixed Color Reagent B	
	Standard	
Microplate Wells	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.*	

*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 500 mL graduated cylinders.
- Test tubes for dilution.
- Human TNF- α Controls (optional; available from R&D Systems).

PRECAUTIONS

Calibrator Diluent RD6-35 contains sodium azide which may react with lead and copper plumbing to form explosive metallic azides. Flush with large volumes of water during disposal.

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 $\leq -20^{\circ}$ C. Avoid repeated freeze-thaw cycles.

Serum - Use a serum separator tube (SST) and allow samples to clot for 30 minutes before centrifugation for 15 minutes at 1000 x g. Remove serum and assay immediately or aliquot and store samples at $\leq -20^{\circ}$ C. Avoid repeated freeze-thaw cycles.

Plasma - Collect plasma using EDTA, heparin, or citrate as an anticoagulant. Centrifuge for 15 minutes at 1000 x g within 30 minutes of collection. Assay immediately or aliquot and store samples at $\leq -20^{\circ}$ C. Avoid repeated freeze-thaw cycles.

REAGENT PREPARATION

Bring all reagents to room temperature before use.

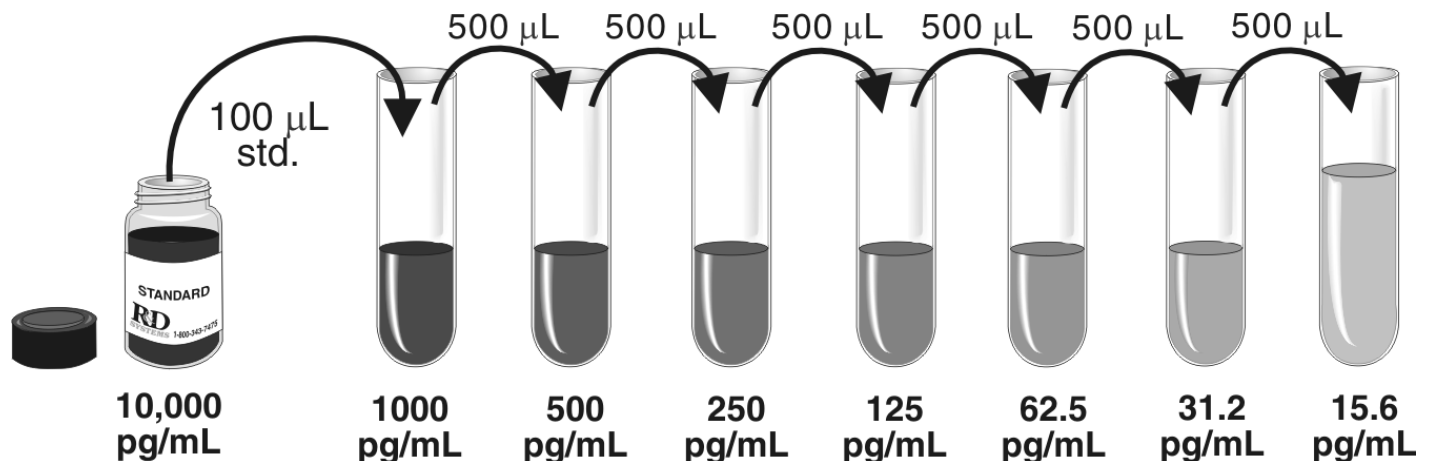
Wash Buffer - If crystals have formed in the concentrate, warm to room temperature and mix gently until the crystals have completely dissolved. Dilute 20 mL of Wash Buffer Concentrate into deionized or distilled water to prepare 500 mL of Wash Buffer.

Calibrator Diluent RD6-35 (1X) (*for cell culture supernate samples*) - Add 20 mL of Calibrator Diluent RD6-35 to 80 mL of deionized or distilled water to yield 100 mL of Diluted Calibrator Diluent RD6-35.

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

TNF- α Standard - Refer to vial label for reconstitution volume. Reconstitute the TNF- α 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 900 μ L of Calibrator Diluent RD6-35 (*for serum/plasma samples*) or Calibrator Diluent RD6-35 (1X) (*for cell culture supernate samples*) into the 1000 pg/mL tube. Pipette 500 μ L of the appropriate Calibrator Diluent into the remaining tubes. 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 appropriate Calibrator Diluent 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 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 Assay Diluent RD1F to each well. Assay Diluent RD1F will have a precipitate present. Mix well before and during use.
4. Add 200 μL of Standard, sample, or control per well. 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 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.
6. Add 200 μL of TNF- α Conjugate to each well. Cover with a new adhesive strip.
For Serum/Plasma Samples: Incubate for 2 hours at room temperature.
For Cell Culture Supernate Samples: Incubate for 1 hour at room temperature.
7. Repeat the aspiration/wash as in step 5.
8. Add 200 μL of Substrate Solution to each well. Incubate for 20 minutes at room temperature. **Protect from light.**
9. 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 if the color change does not appear uniform, 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.

ASSAY PROCEDURE SUMMARY

1. Prepare all reagents and standards as directed.



2. Add 50 μL Assay Diluent RD1F to each well.



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

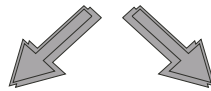


4. Aspirate and wash 4 times.



5. Add 200 μL Conjugate to each well.

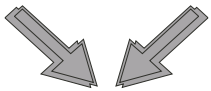
**Serum/Plasma
Samples**



**Cell Culture Supernate
Samples**

Incubate 2 hours at RT.

Incubate 1 hour at RT.



6. Aspirate and wash 4 times.



7. Add 200 μL Substrate Solution to each well.
Incubate 20 minutes at RT. **Protect from light.**



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

CALCULATION OF RESULTS

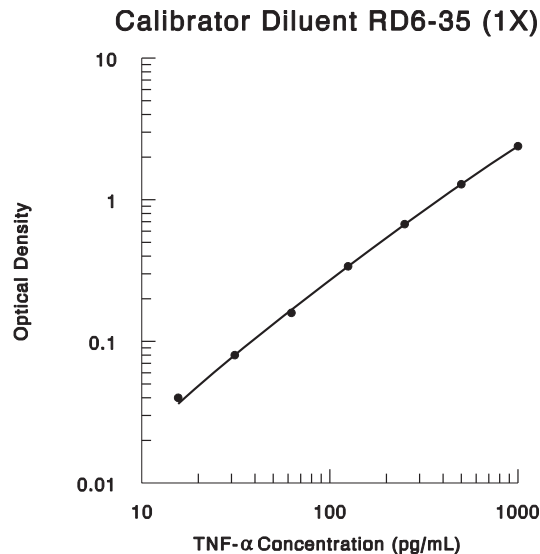
Average the duplicate readings for each standard, control, and sample and subtract the average zero standard optical density.

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 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.

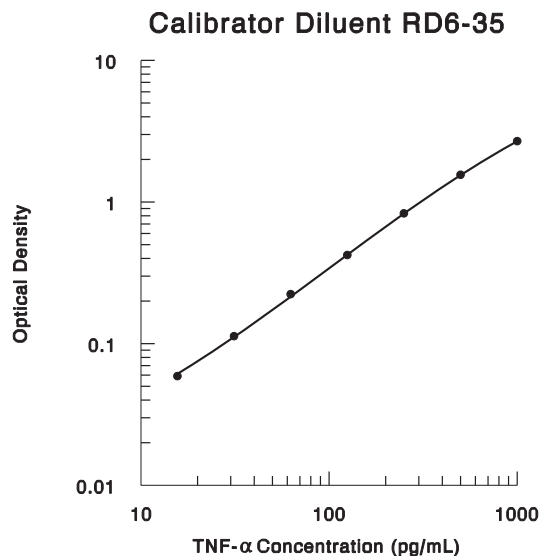
If samples have been diluted, the concentration read from the standard curve must be multiplied by the dilution factor.

TYPICAL DATA

These standard curves are provided for demonstration only. A standard curve should be generated for each set of samples assayed.



(pg/mL)	O.D.	Average	Corrected
0	0.033 0.035 0.071	0.034	—
15.6	0.076 0.112	0.074	0.040
31.2	0.115 0.187	0.114	0.080
62.5	0.199 0.361	0.193	0.159
125	0.384 0.689	0.373	0.339
250	0.725 1.314	0.707	0.673
500	1.326 2.364	1.320	1.286
1000	2.480	2.422	2.388



(pg/mL)	O.D.	Average	Corrected
0	0.045 0.049 0.105	0.047	—
15.6	0.107 0.157	0.106	0.059
31.2	0.162 0.269	0.160	0.113
62.5	0.272 0.469	0.271	0.224
125	0.470 0.872	0.470	0.423
250	0.885 1.586	0.879	0.832
500	1.626 2.729	1.606	1.559
1000	2.746	2.737	2.690

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 the 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.

Serum/Plasma Assay

Sample	Intra-assay Precision			Inter-assay Precision		
	1	2	3	1	2	3
n	20	20	20	20	20	20
Mean (pg/mL)	48.1	317	587	45.8	301	587
Standard deviation	2.5	13.2	27.1	3.4	13.7	31.6
CV (%)	5.2	4.2	4.6	7.4	4.6	5.4

Cell Culture Supernate Assay

Sample	Intra-assay Precision			Inter-assay Precision		
	1	2	3	1	2	3
n	20	20	20	20	20	20
Mean (pg/mL)	41.2	281	546	43.8	283	567
Standard deviation	2.2	12.5	27.3	3.0	24.7	41.5
CV (%)	5.3	4.4	5.0	6.8	8.7	7.3

RECOVERY

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

Sample Type	Average % Recovery	Range
Cell culture media	98	91 - 109%
Serum	107	94 - 119%
EDTA plasma	110	98 - 128%
Heparin plasma	101	89 - 115%
Citrate plasma	106	89 - 123%

LINEARITY

To assess linearity of the assay, five samples were spiked with high concentrations of TNF- α in various matrices and diluted with the appropriate Calibrator Diluent to produce samples with values within the dynamic range of the assay.

		Cell culture media	Serum	EDTA plasma	Heparin plasma	Citrate plasma
1:2	Average % of Expected	98	105	104	99	107
	Range (%)	96 - 102	100 - 113	100 - 108	91 - 103	101 - 114
1:4	Average % of Expected	102	102	105	100	106
	Range (%)	96 - 104	97 - 107	100 - 113	87 - 109	91 - 115
1:8	Average % of Expected	102	100	102	104	117
	Range (%)	98 - 103	96 - 109	93 - 107	95 - 110	109 - 126
1:16	Average % of Expected	104	97	102	108	109
	Range (%)	98 - 108	92 - 102	95 - 112	102 - 111	101 - 121

SENSITIVITY

Twenty-five assays were evaluated and the minimum detectable dose (MDD) of TNF- α ranged from 0.5 - 5.5 pg/mL. The mean MDD was 1.6 pg/mL.

The MDD 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 immunoassay is calibrated against a highly purified *E. coli*-expressed recombinant human TNF- α produced at R&D Systems.

The NIBSC/WHO 2nd International Standard 88/786 (natural human TNF- α) was evaluated in this kit. The dose response curve of this International Standard parallels the Quantikine standard curve. To convert sample values obtained with the Quantikine Human TNF- α kit to approximate NIBSC 88/786 international units, use the equation below.

NIBSC (88/786) approximate value (IU/mL) = 0.050 x Quantikine Human TNF- α value (pg/mL).

Note: *Based on data generated in November 2008.*

SAMPLE VALUES

Serum/Plasma - Forty serum and plasma samples from apparently healthy volunteers were evaluated for the presence of TNF- α in this assay. No medical histories were available for the donors used in this study. All samples measured less than the lowest standard, 15.6 pg/mL.

Cell Culture Supernates - Human peripheral blood mononuclear cells (1×10^6 cells/mL) were cultured in RPMI supplemented with 10% fetal calf serum, 50 μ M β -mercaptoethanol, 2 mM L-glutamine, 100 U/mL penicillin, and 100 μ g/mL streptomycin sulfate. Cells were stimulated for 1, 3, and 5 days. Aliquots of the culture supernate were removed on days 1, 3, and 5 and assayed for levels of natural TNF- α .

Stimulant(s)	Day 0 (pg/mL)	Day 1 (pg/mL)	Day 3 (pg/mL)	Day 5 (pg/mL)
10 μ g/mL PHA	ND	3714	6740	7239
10 μ g/mL PHA + 10 ng/mL rhIL-2	ND	3326	5981	7205
50 ng/mL PMA	ND	1442	1274	2000
50 ng/mL LPS	ND	3045	1489	1233

ND = Non-detectable

SPECIFICITY

This assay recognizes both natural and recombinant human TNF- α . The factors listed below were prepared at 50 ng/mL in Calibrator Diluent RD6-35 and assayed for cross reactivity. Preparations of the following factors at 50 ng/mL in a mid-range rhTNF- α control were assayed for interference. No significant cross-reactivity or interference was observed.

Recombinant human:

ANG
 β -ECGF
EGF
FGF acidic
FGF basic
FGF-4
G-CSF
GM-CSF
GRO α
IGF-I
IGF-II
IFN- γ
IL-1 α
IL-1 β
IL-1ra
IL-2
IL-3
IL-4
IL-5
IL-6
IL-6 sR
IL-7

IL-8
IL-9
IL-10
IL-11
LIF
M-CSF
MCP-1
MIP-1 α
MIP-1 β
OSM
PDGF-AA
PDGF-AB
PDGF-BB
RANTES
SLPI
TGF- α
TGF- β 1
TGF- β 2
TGF- β 3
TNF- β
sTNF RI
sTNF RII

Recombinant mouse:

EGF
GM-CSF
IL-1 α
IL-1 β
IL-3
IL-4
IL-5
IL-6
IL-7
IL-9
MIP-1 α
MIP-1 β
SCF

Other recombinants:
rat IL-1ra
amphibian TGF- β 5
chicken TGF- β 3
equine TNF- α
porcine TNF- α
rhesus macaque TNF- α

Natural proteins:

bovine FGF acidic
bovine FGF basic
human PDGF
porcine PDGF
human TGF- β 1
porcine TGF- β 1
porcine TGF- β 1.2
porcine TGF- β 2

Less than 1% cross-reactivity was observed with recombinant canine, mouse, and rat TNF- α .

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PLATE LAYOUT

Use this plate layout to record standards and samples assayed.

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