Quantikine® ELISA

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.

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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).

Human 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 177 aa extracellular domain (ECD) (12, 13). Within the ECD, human TNF-α shares 97% aa sequence identity with rhesus monkey, and 71-92% aa identity with bovine, canine, cotton rat, equine, feline, mouse, porcine, and rat 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 (14). 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 (15, 16). Shedding of membrane bound TNF-α by TACE/ADAM17 releases the bioactive cytokine, a 55 kDa soluble trimer of the TNF-α extracellular domain (17-19).

TNF-α binds the ubiquitous 55-60 kDa TNF RI (20, 21) and the hematopoietic cell-restricted 78-80 kDa TNF RII (22, 23), both of which are also expressed as homotrimers (1, 24). Both type I and type II receptors bind TNF-α with comparable affinity and can promote NFkB activation (25-28). Only TNF RI, however, contains a cytoplasmic death domain which triggers the activation of apoptosis (3, 29). Soluble forms of both types of receptors are released into human serum and urine and can neutralize the biological activity of TNF-α (30-32).

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 this 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.
- Variations in sample collection, processing, and storage may cause sample value differences.
- 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.

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

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

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.

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 of standards.
- 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 protective gloves, clothing, eye, and face protection. Wash hands thoroughly after handling.

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, 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 ≤ -20 °C. Avoid repeated freeze-thaw cycles.

REAGENT PREPARATION

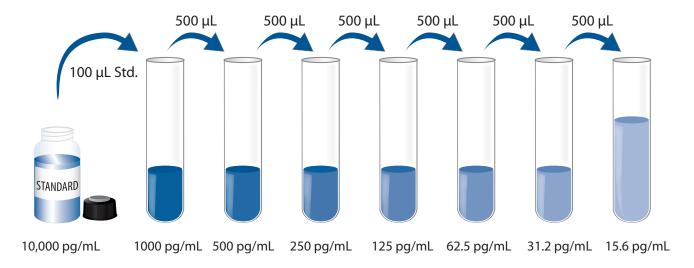**Bring all reagents to room temperature before use.**

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 10 mL of Calibrator Diluent RD6-35 to 40 mL of deionized or distilled water to yield 50 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 μL 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 μL 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 μL 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).

| 500 μL | 500 μL | 500 μL | 500 μL | 500 μL | 500 μL |

100 μL Std.

| 10,000 pg/mL | 1000 pg/mL | 500 pg/mL | 250 pg/mL | 125 pg/mL | 62.5 pg/mL | 31.2 pg/mL | 15.6 pg/mL |

ASSAY PROCEDURE

Bring all reagents and samples to room temperature before use. It is recommended that all standards, samples, 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 Cell Culture Supernate Samples: Incubate for 1 hour at room temperature.
   For Serum/Plasma Samples: Incubate for 2 hours 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.

CALCULATION OF RESULTS

Average the duplicate readings for each standard, control, and sample and subtract the average zero standard optical density.
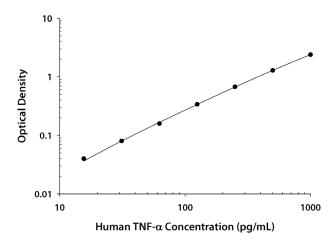
Create a standard curve by reducing the data using computer software capable of generating a four parameter logistic (4-PL) curve fit. As an alternative, construct a standard curve by plotting the mean absorbance for each standard on the y-axis against the concentration on the x-axis and draw a best 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.
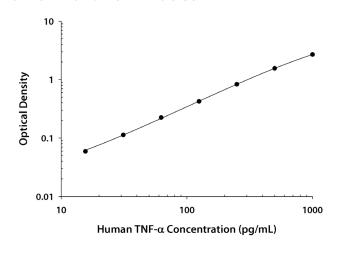
TYPICAL DATA

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

CALIBRATOR DILUENT RD6-35 (1X)

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

CALIBRATOR DILUENT RD6-35

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

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.

CELL CULTURE SUPERNATE ASSAY

	Intra-Assay Precision			Inter-Assay Precision		
Sample	**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

SERUM/PLASMA ASSAY

	Intra-Assay Precision			Inter-Assay Precision		
Sample	**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

RECOVERY

The recovery of TNF-α 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=5)	98	91-109%
Serum (n=5)	107	94-119%
EDTA plasma (n=5)	110	98-128%
Heparin plasma (n=5)	101	89-115%
Citrate plasma (n=5)	106	89-123%

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.

LINEARITY

To assess linearity of the assay, 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 (n=5)	Serum (n=5)	EDTA plasma (n=5)	Heparin plasma (n=5)	Citrate plasma (n=5)
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

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 x 10^6 cells/mL) were cultured in RPMI supplemented with 10% fetal calf serum, 50 μM β-mercaptoethanol, 2 mM L-glutamine, 100 U/mL penicillin, and 100 μg/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 μg/mL PHA	ND	3714	6740	7239
10 μg/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 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	IL-8
β-ECGF	IL-9
EGF	IL-10
FGF acidic	IL-11
FGF basic	LIF
FGF-4	M-CSF
G-CSF	MCP-1
GM-CSF	MIP-1α
GROα	MIP-1β
IGF-I	OSM
IGF-II	PDGF-AA
IFN-γ	PDGF-AB
IL-1α	PDGF-BB
IL-1β	RANTES
IL-1ra	SLPI
IL-2	TGF-α
IL-3	TGF-β1
IL-4	TGF-β2
IL-5	TGF-β3
IL-6	TNF-β
IL-6 sR	sTNF RI
IL-7	sTNF RII

Recombinant mouse:

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

Other recombinants:

rat IL-1ra
amphibian TGF-β5
chicken TGF-β3
equine TNF-α
porcine TNF-α
rhesus macaque TNF-α

Natural proteins:

bovine FGF acidic
bovine FGF basic
human PDGF
porcine PDGF
human TGF-β1
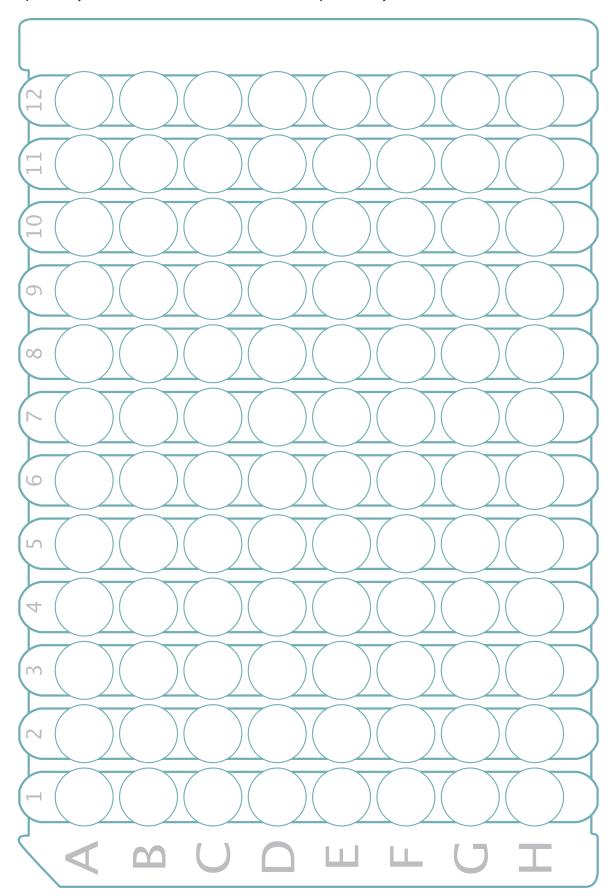porcine TGF-β1
porcine TGF-β1.2
porcine TGF-β2

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.

For research use only. Not for use in diagnostic procedures.

NOTES

NOTES