Quantikine® HS ELISA

Human TNF-α Immunoassay

Catalog Number HSTA00D
SSTA00D
PHSTA00D

For the quantitative determination of human Tumor Necrosis Factor alpha (TNF-α) concentrations in 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

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 NFκB 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 HS Human TNF-α Immunoassay is a 6.5 hour solid phase ELISA designed to measure human TNF-α in serum and plasma. It contains *E. coli*-derived recombinant human TNF-α and antibodies raised against the recombinant factor. It has been shown to accurately quantitate recombinant human TNF-α. Results obtained with naturally occurring TNF-α samples showed linear curves that were parallel to the standard curves obtained using the recombinant 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

DUE TO THE HIGH SENSITIVITY OF THIS KIT, PLEASE NOTE THE FOLLOWING PRECAUTIONS.

- **Alkaline phosphatase is detectable in saliva.** Take precautionary measures (*e.g. wear a mask and gloves*) to protect reagents during preparation and use and while running the assay (reagent preparation through the addition of Stop Solution).
- Inorganic phosphate is a strong competitive inhibitor of alkaline phosphatase; avoid the use of PBS based wash buffers and other sources of inorganic phosphate contamination.
- Use separate pipettes and glassware for dispensing all reagents to avoid cross-contamination.
- Careful washing of the microplate is essential to minimize nonspecific binding of the conjugate.

This assay employs the quantitative sandwich enzyme immunoassay technique. A monoclonal antibody specific for human 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 human 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. After an incubation period, an amplifier 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.
- Although this kit has been designed to eliminate matrix problems, there may exist some samples that give falsely elevated values when assayed neat. If samples generate values that are suspiciously high or higher than the highest standard, dilute the samples in 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 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

- To ensure accurate results, bring liquids to room temperature and mix to homogeneity prior to pipetting or aliquoting.
- When mixing 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.
- Neither the addition of the Substrate Solution nor the Stop Solution will result in a color change.
- Addition of the Amplifier Solution will result in a color change (colorless to shades of maroon).
- Amplifier Solution and Stop Solution should be added to the plate in the same order as 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 # HSTA00D	CATALOG # SSTA00D	DESCRIPTION	STORAGE OF OPENED/ RECONSTITUTED MATERIAL
Human TNF-α HS Microplate	890953	1 plate	6 plates	96 well polystyrene microplate (12 strips of 8 wells) coated with a monoclonal antibody specific for human 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.*
Human TNF-α HS Standard	893154	1 vial	6 vials	Recombinant human TNF-α in a buffered protein base with preservatives; lyophilized. *Refer to the vial label for reconstitution volume.*	Aliquot and store at ≤ -70 °C for up to 1 month* in a manual defrost freezer. Avoid multiple freeze-thaw cycles.
Human TNF-α HS Conjugate	893153	1 vial	6 vials	21 mL/vial of polyclonal antibody specific for human TNF-α conjugated to alkaline phosphatase with preservatives. *May contain a precipitate. Mix well before and during use.*	Store for up to 1 month at 2-8 °C.*
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 RD6-13	895491	1 vial	6 vials	21 mL/vial of a buffered protein base with preservatives.	
Wash Buffer Concentrate	895188	1 vial	6 vials	100 mL/vial of a 10-fold concentrated solution of buffered surfactant with preservative. *May turn yellow over time.*	
Stop Solution	895032	1 vial	6 vials	6 mL/vial of 2 N sulfuric acid.	
Substrate	895884	1 vial	6 vials	Lyophilized NADPH with stabilizers.	**Store in an upright position** for up to 1 month at ≤ -20 °C in a manual defrost freezer.* Avoid repeated freeze-thaw cycles.
Substrate Diluent	895885	1 vial	6 vials	7 mL/vial of buffered solution with stabilizers.	
Amplifier	895886	1 vial	6 vials	Lyophilized amplifier enzymes with stabilizers.	
Amplifier Diluent	895887	1 vial	6 vials	7 mL/vial of buffered solution containing INT-violet with stabilizer.	
Plate Sealers	N/A	8 strips	48 strips	Adhesive strips.	

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

HSTA00D contains sufficient materials to run an ELISA on one 96 well plate.
SSTA00D (SixPak) contains sufficient materials to run ELISAs on six 96 well plates.

This kit is also available in a PharmPak (R&D Systems, Catalog # PHSTA00D). 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 490 nm, with dual wavelength correction set at 650 nm or 690 nm.
- Pipettes and pipette tips.
- Deionized or distilled water.
- Squirt bottle, manifold dispenser, or automated microplate washer.
- 1000 mL graduated cylinder.
- **Polypropylene** test tubes for dilution of standards.
- Human TNF-α Controls (optional; available from R&D Systems).

PRECAUTIONS

Some components in this kit contain sodium azide which may react with lead and copper plumbing to form explosive metallic azides. Flush with large volumes of water during disposal.

Alkaline phosphatase and TNF-α are detectable in saliva. Take precautionary measures (e.g. wear a mask and gloves) to protect reagents during preparation and use and while running the assay (reagent preparation through the addition of Stop Solution).

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

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

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.

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.

Note: *Allowing samples to sit on the clot for more than 30 minutes may result in higher TNF-α levels.*

Plasma - Collect plasma using EDTA, heparin, or citrate as an anticoagulant. Centrifuge for 15 minutes at 1000 x g within 30 minutes of collection. Remove plasma and assay immediately or aliquot and store samples at ≤ -20 °C. Avoid repeated freeze-thaw cycles.

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REAGENT PREPARATION

Bring all reagents to room temperature before use.

Note: *Alkaline phosphatase and TNF-α are detectable in saliva. Take precautionary measures (e.g. wear a mask and gloves) to protect reagents during preparation and use and while running the assay (reagent preparation through the addition of Stop Solution).*

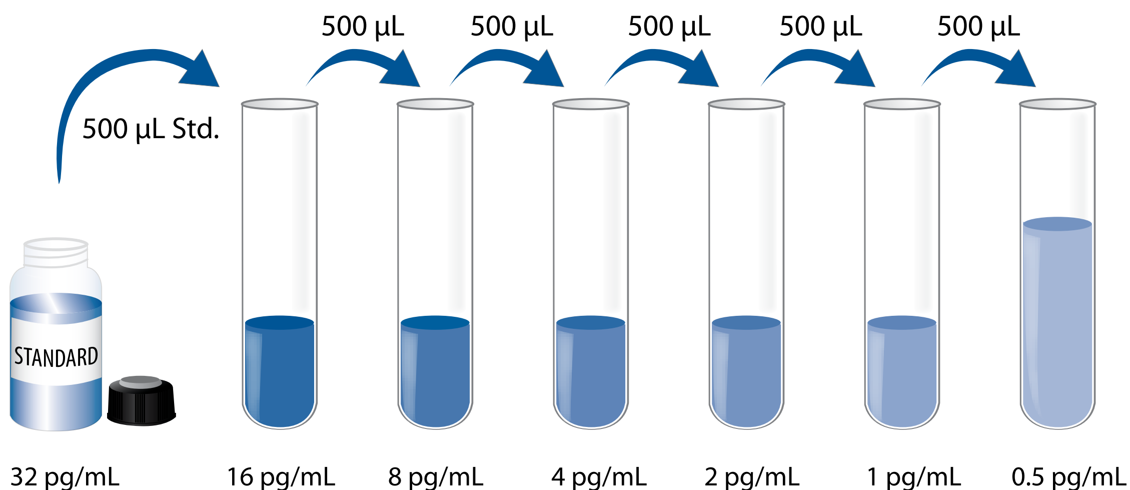
Wash Buffer - If crystals have formed in the concentrate, warm to room temperature and mix gently until the crystals have completely dissolved. Add 100 mL of Wash Buffer Concentrate to deionized or distilled water to prepare 1000 mL of Wash Buffer.

Substrate Solution - Reconstitute the lyophilized Substrate with 6.0 mL of Substrate Diluent at least 10 minutes before use. **Re-stopper and re-cap the vial**, and mix thoroughly. Avoid contamination.

Amplifier Solution - Reconstitute the lyophilized Amplifier with 6.0 mL of Amplifier Diluent at least 10 minutes before use. **Re-stopper and re-cap the vial**, and mix thoroughly. Avoid contamination.

Human TNF-α HS Standard - **Refer to the vial label for reconstituion volume.** Reconstitute the Human TNF-α HS Standard with Calibrator Diluent RD6-13. This reconstitution produces a stock solution of 32 pg/mL. Allow the standard to sit for a minimum of 15 minutes with gentle agitation prior to making dilutions.

Use polypropylene tubes. Pipette 500 µL of Calibrator Diluent RD6-13 into each tube. Use the stock solution to produce a dilution series (below). Mix each tube thoroughly before the next transfer. The undiluted standard serves as the high standard (32 pg/mL). Calibrator Diluent RD6-13 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, samples, and controls be assayed in duplicate.

Note: *Alkaline phosphatase and TNF-α are detectable in saliva. Take precautionary measures to protect reagents (e.g. wear a mask and gloves).*

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 Assay Diluent RD1F to each well. *Assay Diluent RD1F contains a precipitate. 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 3 hours at room temperature. A plate layout is provided to record standards and samples assayed.
5. Wash
   a. Remove liquid from the wells by aspirating or inverting the plate and decanting the contents.
   b. Remove excess liquid by grasping the plate firmly and smartly rapping the inverted plate on a clean paper towel at least 5 times.
   c. Fill each well with 400 µL of Wash Buffer using a squirt bottle, manifold dispenser, or autowasher.
   d. Remove liquid from the wells by aspirating or inverting the plate and decanting the contents.
   e. Repeat steps b, c, and d 5 times for a total of 6 washes. After the last wash, smartly rap the inverted plate on a clean paper towel at least 10 times to remove excess Wash Buffer.
6. Add 200 µL of Human TNF-α HS Conjugate to each well. *The TNF-α HS Conjugate may contain a precipitate. Mix well before and during use.* Cover with a new adhesive strip. Incubate for 2 hours at room temperature.
7. Repeat the wash as in step 5.
8. Add 50 µL of Substrate Solution to each well. Cover with a new adhesive strip. Incubate for 1 hour at room temperature. **Do not wash the plate.**
9. Add 50 µL of Amplifier Solution to each well. Cover with a new adhesive strip. Incubate for 30 minutes at room temperature. **Note:** *Addition of Amplifier Solution initiates color development.*
10. Add 50 µL of Stop Solution to each well. Addition of Stop Solution does not affect color in the wells.
11. Determine the optical density of each well within 30 minutes, using a microplate reader set to 490 nm. If wavelength correction is available, set to 650 nm or 690 nm. If wavelength correction is not available, subtract readings at 650 nm or 690 nm from the readings at 490 nm. This subtraction will correct for optical imperfections in the plate. Readings made directly at 490 nm without correction may be higher and less accurate.

CALCULATION OF RESULTS

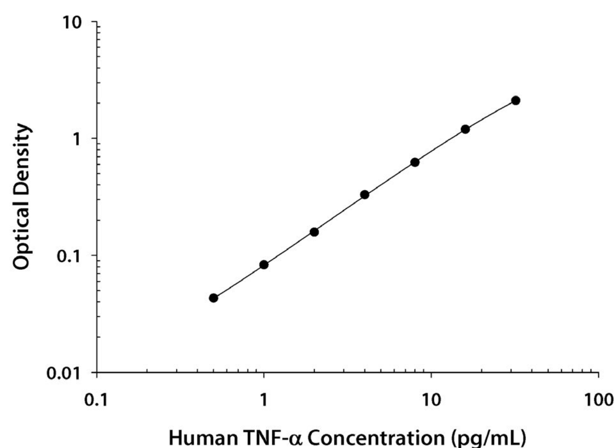
Average the duplicate readings for each standard, control, and sample and subtract the average zero standard optical density (O.D.).

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-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

The following 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	0.091 0.108	0.100	—
0.5	0.139 0.147	0.143	0.043
1	0.176 0.189	0.183	0.083
2	0.241 0.275	0.258	0.158
4	0.409 0.451	0.430	0.330
8	0.694 0.753	0.724	0.624
16	1.252 1.332	1.292	1.192
32	2.193 2.214	2.204	2.104

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 forty-one 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	41	41	41
Mean (pg/mL)	1.96	11.5	22.1	1.83	10.5	20.3
Standard deviation	0.17	0.49	0.68	0.19	0.76	1.50
CV (%)	8.7	4.3	3.1	10.4	7.2	7.4

RECOVERY

The recovery was determined by spiking samples with human TNF-α to three different levels throughout the range of the assay in various matrices.

Sample Type	Average % Recovery	Range
Serum (n=4)	93	85-98%
EDTA plasma (n=4)	99	92-111%
Heparin plasma (n=4)	93	87-103%
Citrate plasma (n=4)	95	88-105%

SENSITIVITY

Seventy-four assays were evaluated and the minimum detectable dose (MDD) of TNF-α ranged from 0.038-0.191 pg/mL. The mean MDD was 0.106 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 human TNF-α and diluted with Calibrator Diluent to produce samples with values within the dynamic range of the assay.

		Serum (n=4)	EDTA plasma (n=4)	Heparin plasma (n=4)	Citrate plasma (n=4)
1:2	Average % of Expected	104	98	100	104
	Range (%)	97-107	92-102	97-106	97-109
1:4	Average % of Expected	108	101	98	102
	Range (%)	97-115	98-104	91-103	102-103
1:8	Average % of Expected	106	104	102	101
	Range (%)	97-114	100-109	95-106	95-104
1:16	Average % of Expected	100	99	101	104
	Range (%)	97-105	93-107	95-108	95-110

CALIBRATION

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

The NIBSC/WHO TNF-α 2nd International Reference Standard 88/786, which is intended as a potency standard, was evaluated in this kit. This standard is an *E. coli*-expressed recombinant human TNF-α.

The dose response curve of this 2nd International Standard parallels the Quantikine HS standard curve. To convert sample values obtained with the Quantikine HS Human TNF-α kit to approximate NIBSC 88/786 nominally assigned mass values, use the equation below.

NIBSC/WHO (88/786) approximate value (IU/mL) = 0.086 x Quantikine HS Human TNF-α value (pg/mL).

SAMPLE VALUES

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.

Sample Type	Mean of Detectable (pg/mL)	% Detectable	Range (pg/mL)
Serum (n=33)	1.206	100	0.550-2.816
EDTA plasma (n=22)	1.036	91	ND-2.139
Heparin plasma (n=26)	0.873	85	ND-1.411
Citrate plasma (n=14)	0.739	79	ND-1.195

ND=Non-detectable

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SPECIFICITY

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

Recombinant human:

ANG
Ang-2
AR
BDNF
CD4
CD40
CD40 Ligand
CNTF
CT-1
CTLA-4
Epo
Fas
GDNF
GITR
IFN-γ
IL-1α
IL-1β
IL-1ra
IL-2
IL-3
IL-4
IL-5
IL-6
IL-7
IL-8
IL-10
IL-11
IL-12
IL-12 p40
IL-13
IL-15
IL-16
IL-17
Leptin
LIF
MIF
NT-3
NT-4
OPG
OSM
PTN
SCF
SLPI
SMDF
TNF-β
TNF RI
TNF RII
Tpo
TRAIL
TRANCE

Recombinant mouse:

CT-1
CTLA-4
Fas
Fas Ligand
GITR Ligand
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-11
IL-12
IL-12 p40
IL-13
IL-17
Leptin
LIF
OPG
OPN
OSM
SCF
TNF-α
Tpo
TRANCE

Recombinant rat:

CNTF
GDNF
IFN-γ
IL-1α
IL-1β
IL-2
IL-4
IL-6
IL-10
Leptin
MK
TNF-α

Recombinant porcine:

IL-1α
IL-1β
IL-2
IL-4
IL-6
IL-8
IL-10
TNF-α

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PLATE LAYOUT

Use this plate layout to record standards and samples assayed.

NOTES

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