Quantikine® ELISA

Human TGF-α Immunoassay

Catalog Number DTGA00

For the quantitative determination of human Transforming Growth Factor alpha (TGF-α) concentrations in cell culture supernates, serum, plasma, and human milk.

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

Transforming growth factor alpha (TGF-α) was originally isolated as one of two factors actively secreted from virally transformed fibroblasts. Subsequent exposure of normal fibroblasts to this factor elicited a reversible transformed or malignant phenotype (1). Originally named sarcoma growth factor, TGF-α was later identified, cloned, and placed in the epidermal growth factor (EGF) family of cytokines (1-4). Analysis of the promotor region of the gene suggests that transcription is influenced by EGF, estrogen, glucocorticoids, protein kinase C (PKC), retinoic acid, and thyroid hormone (4). The human TGF-α gene includes six exons which encode a 160 amino acid (aa) precursor protein. The precursor contains an amino-terminal, plasma membrane-targeting signal sequence, extracellular domain possessing N- and O-linked glycosylation sites, a single transmembrane-spanning region, and a cytoplasmic tail (3, 4). The mouse and rat homologues of human TGF-α have since been cloned and are approximately 93% identical to the human sequence at the aa level, suggesting a high degree of evolutionary conservation (4).

The mature, soluble form of TGF-α is a 50 aa section at the carboxy-terminal end of the extracellular domain. This region contains several highly conserved cysteine residues, which participate in intramolecular disulfide bonding to generate a series of loops. This three-dimensional structure, now known as the EGF-like motif, is critical to the achievement of high affinity binding to its receptor and is common to all members of the EGF family (3, 4). Shedding of TGF-α from its integral membrane precursor is highly regulated. Adenosine triphosphate (ATP), calcium, EGF receptor (EGF R) activation, G-protein signaling, matrix metalloproteinases and PKC have all been implicated in the regulatory mechanisms of soluble TGF-α release (3, 4). Evidence suggests that tumor necrosis factor alpha converting enzyme (TACE), a member of the disintegrin and metalloproteinase (ADAM) family, is the enzyme responsible for cleaving mature TGF-α from its precursor (5). Once released, mature TGF-α has been described as an approximately 5-30 kDa protein due to variable glycosylation (3, 4). Soluble TGF-α acts in autocrine and paracrine fashions via high affinity binding to its receptor, EGF R (4). Receptor-ligand binding elicits EGF R dimerization, tyrosine autophosphorylation, and activation of signal transduction cascades involving proteins possessing the Src homology domain that eventually impact transcription (4).

TGF-α expression is characterized by a relatively widespread distribution. While TGF-α was first described in embryonic and neoplastic cells, it has since been described in the normal adult endocrine, hematopoietic, immune, integumentary, nervous, respiratory, and urinary systems (4, 6). TGF-α expression is exaggerated in transformed cells and tumor tissues of many types (4, 6). Although TGF-α is most often associated with its proliferation, differentiation, and transformation-promoting effects, it is also involved in angiogenesis, bone resorption, cell metabolism, cell migration and wound healing. Further, TGF-α has been implicated not only in myriad forms of cancer, but also in several neurodegenerative disorders (4, 6).

The Quantikine® Human TGF-α Immunoassay is a 4.5 hour solid-phase ELISA designed to measure human TGF-α in cell culture supernates, serum, plasma, and human milk. It contains *E. coli*-expressed recombinant human TGF-α and has been shown to accurately quantitate the recombinant factor. Results obtained using natural human TGF-α 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 human TGF-α.

PRINCIPLE OF THE ASSAY

This assay employs the quantitative sandwich enzyme immunoassay technique. A polyclonal antibody specific for human TGF-α has been pre-coated onto a microplate. Standards and samples are pipetted into the wells and any TGF-α present is bound by the immobilized antibody. After washing away any unbound substances, an enzyme-linked polyclonal antibody specific for human TGF-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 TGF-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.
- If samples generate values higher than the highest standard, 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.
- 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
Human TGF-α Microplate	892356	96 well polystyrene microplate (12 strips of 8 wells) coated with a polyclonal antibody specific for human TGF-α.	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 TGF-α Conjugate	892357	21 mL of a polyclonal antibody specific for human TGF-α conjugated to horseradish peroxidase with preservatives.	May be stored for up to 1 month at 2-8 °C.*
Human TGF-α Standard	892358	Recombinant human TGF-α in a buffered protein base with preservatives; lyophilized. *Refer to the vial label for reconstitution volume.*	
Assay Diluent RD1W	895117	11 mL of a buffered protein base with preservatives.	
Calibrator Diluent RD5-24	895325	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	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.

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.
- Human TGF-α Controls (optional; R&D Systems®, Catalog # QC168).

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 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.*

Human Milk - Centrifuge for 15 minutes at 1000 x g at 2-8 °C. Collect the aqueous fraction and centrifuge twice more for a total of 3 times. Assay immediately or aliquot and store samples at ≤ -20 °C. Avoid repeated freeze-thaw cycles.

REAGENT PREPARATION

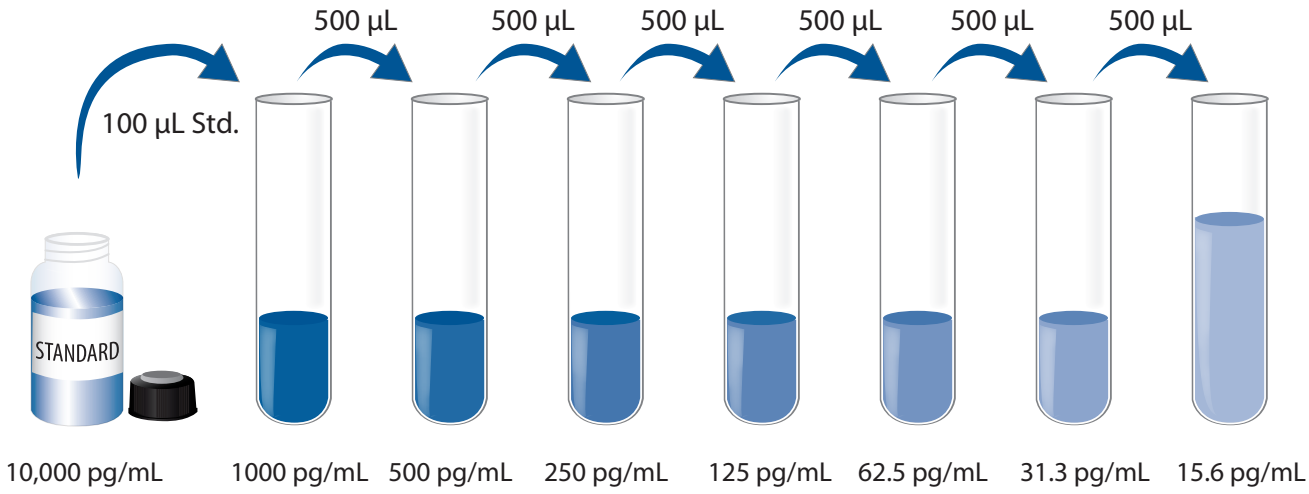
Bring all reagents to room temperature before use.

Wash Buffer - If crystals have formed in the concentrate, warm to room temperature and mix gently until the crystals have completely dissolved. 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. 200 µL of the resultant mixture is required per well.

Human TGF-α Standard - **Refer to the vial label for reconstitution volume.** Reconstitute the Human TGF-α Standard with deionized or distilled water. This reconstitution produces a stock solution of 10,000 pg/mL. Mix the standard to ensure complete reconstitution and allow the standard to sit for a minimum of 15 minutes with gentle agitation prior to making dilutions.

Pipette 900 µL of Calibrator Diluent RD5-24 into the 1000 pg/mL tube. Pipette 500 µL 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. Calibrator Diluent RD5-24 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, samples, and controls be assayed in duplicate.

1. Prepare all reagents, working standards, 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 100 µL of Assay Diluent RD1W to each well.
4. Add 50 µL of standard, control, or sample per well. Cover with the adhesive strip provided. Incubate for 2 hours at room temperature.
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 Human TGF-α 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 200 µL of Substrate Solution to each well. Incubate for 30 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 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

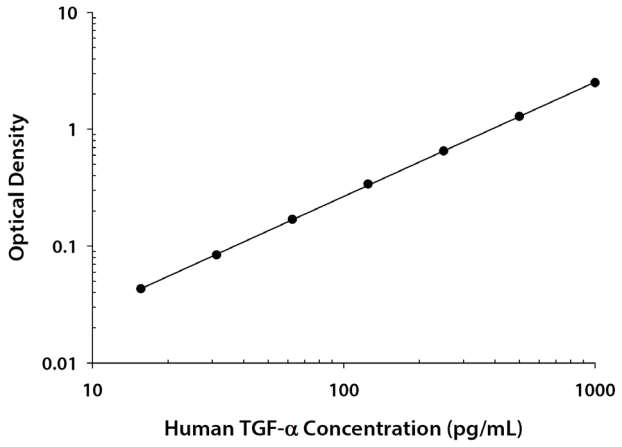
Average the duplicate readings for each standard, control, and sample and subtract the average zero standard optical density (O.D.).

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 human TGF-α concentrations versus the log of the O.D. on a linear scale, and the best fit line can be determined by regression analysis.

If the 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	0.025 0.025	0.025	—
15.6	0.066 0.069	0.068	0.043
31.3	0.109 0.109	0.109	0.084
62.5	0.191 0.196	0.194	0.169
125	0.362 0.368	0.365	0.340
250	0.661 0.688	0.675	0.650
500	1.311 1.313	1.312	1.287
1000	2.469 2.577	2.523	2.498

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 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	40	40	40
Mean (pg/mL)	91.7	215	419	93.1	221	415
Standard deviation	3.8	9.6	11.1	6.3	10.7	18.1
CV (%)	4.1	4.5	2.6	6.8	4.8	4.4

RECOVERY

The recovery of human TGF-α spiked to levels throughout the range of the assay in various matrices was evaluated.

Sample Type	Average % Recovery	Range
Cell culture media (n=4)	97	92-108%
Serum (n=4)	103	96-114%
EDTA plasma (n=4)	99	88-114%
Heparin plasma (n=4)	103	88-114%

LINEARITY

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

		Cell culture media (n=4)	Serum (n=4)	EDTA plasma (n=4)	Heparin plasma (n=4)	Human milk (n=2)
1:2	Average % of Expected	110	107	105	107	107
	Range (%)	104-114	105-110	103-110	105-111	104-109
1:4	Average % of Expected	109	107	104	106	101
	Range (%)	104-111	104-109	95-109	102-108	96-106
1:8	Average % of Expected	107	106	103	103	100
	Range (%)	100-113	103-107	95-107	98-105	97-102
1:16	Average % of Expected	103	100	96	103	93
	Range (%)	93-111	93-105	90-103	101-104	93-93

SENSITIVITY

Thirty-six assays were evaluated and the minimum detectable dose (MDD) of human TGF-α ranged from 0.55-7.10 pg/mL. The mean MDD was 2.27 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 human TGF-α produced at R&D Systems®.

SAMPLE VALUES

Serum - Samples from apparently healthy volunteers were evaluated for the presence of human TGF-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=61)	21.9	30	ND-32

ND-Non-detectable

Plasma - Thirty-five samples were evaluated for the presence of human TGF-α in this assay. All samples read below the lowest standard, 15.6 pg/mL.

Human Milk - Two aliquots of the same sample were assayed for human TGF-α and measured 171 pg/mL and 176 pg/mL, respectively.

Cell Culture Supernates:
Human peripheral blood cells (1 x 10^6 cells/mL) were cultured in DMEM supplemented with 5% fetal bovine serum, 50 µM β-mercaptoethanol, 2 mM L-glutamine, 100 U/mL penicillin, and 100 µg/mL streptomycin sulfate. Cells were cultured unstimulated or stimulated with 10 µg/mL PHA. Aliquots of the cell culture supernates were removed and assayed for levels of human TGF-α.

Condition	Day 1 (pg/mL)	Day 5 (pg/mL)
Unstimulated	ND	ND
Stimulated	ND	47.2

ND=Non-detectable

HT-29 human colon adenocarcinoma cells were cultured in McCoy's 5a media supplemented with 10% fetal bovine serum and 2 mM L-glutamine. An aliquot of the cell culture supernate was removed, assayed for human TGF-α, and measured 17.3 pg/mL.

SPECIFICITY

This assay recognizes natural and recombinant human TGF-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 TGF-α control were assayed for interference. No significant cross-reactivity or interference was observed.

Recombinant human:

Activin A
Activin RIA
Activin RIIA
Activin RIIB
BMPR-IA
BMPR-IB
BMP-2
BMP-4
BMP-5
BMP-6
CTGF
β-ECGF
EGF
FGF acidic
FGF basic
FGF-4
FGF-5
FGF-6
FGF-9
FGF-10
FGF-18
Flt-3/Flk-2 ligand
Flt-4
$Follistatin_{288}$
$Follistatin_{300}$
$Follistatin_{315}$
G-CSF
GM-CSF
HB-EGF
HGF
HRG-α
HRG-β1
HRG-2
IGF-I
IGF-II
Inhibin A
Inhibin B
KGF (FGF-7)
LAP
M-CSF
MSP
MSPβ
β-NGF
PDGF-AA
PDGF-AB
PDGF-BB
PD-ECGF
PlGF
PlGF-2
TGF-β1
TGF-β1.2
TGF-β2
TGF-β3
TGF-β RII
$VEGF_{121}$
$VEGF_{165}$
VEGF-D

Recombinant mouse:

BMPR-IA
BMPR-IB
FGF-8b
FGF-8c
Flt-3/Flk-2 ligand
G-CSF
GM-CSF
M-CSF
$VEGF_{120}$
$VEGF_{164}$

Recombinant rat:

Agrin
GM-CSF
β-NGF
PDGF-BB

Recombinant porcine:

GM-CSF
TGF-β2

Natural proteins:

bovine FGF acidic
bovine FGF basic
human PDGF
porcine PDGF
porcine TGF-β1
rat TGF-β5

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