

Quantikine[®] HS

Human TNF- α Immunoassay

Catalog Number HSTA50

For the quantitative determination of human tumor necrosis factor alpha (TNF- α) in serum, plasma, and urine.

FOR RESEARCH USE ONLY.
NOT FOR USE IN DIAGNOSTIC PROCEDURES.

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INTRODUCTION

Tumor necrosis factor α (TNF- α) (1, 2), also known as cachectin, and tumor necrosis factor β (TNF- β) (3, 4), also known as lymphotoxin, are two closely related proteins (about 34% amino acid residue homology) that bind to the same cell surface receptors and produce a vast range of similar, but not identical, effects. In contrast to the similarity of their biological activities, the regulation of the expression and processing of the two factors is quite different (5, 6). TNF- α is produced by neutrophils, activated T and B lymphocytes, NK cells, LAK cells, astrocytes, endothelial cells, smooth muscle cells, and some transformed cells (5, 6). TNF- β is produced by lymphocytes (5, 6). The properties and activities of the TNFs have been the subject of numerous reviews (5 - 11).

Mature human TNF- α is a polypeptide of 157 amino acid residues (mouse, rat, or rabbit TNF- α is one amino acid shorter) (5). The apparent molecular weight of human TNF- α under denaturing conditions is approximately 17 kDa (12). Human TNF- α , in contrast to TNF- β , shows no N-glycosylation (mouse TNF- α is N-glycosylated) (5). The biologically active native forms of both TNF- α and TNF- β are trimers (13, 14).

TNF- α , unlike TNF- β , does not possess a typical signal peptide sequence. TNF- α is, however, initially synthesized as a larger protein with the mature 17 kDa factor comprising the C-terminal portion of this precursor. The N-terminal sequence of the precursor contains both hydrophilic and hydrophobic domains and its presence results in the occurrence of TNF- α as a membrane-bound form from which the mature factor is released by proteolytic cleavage (15 - 17). Evidence suggests that the membrane-anchored form of TNF- α on the surface of macrophages and/or monocytes, in addition to serving as a reservoir for release of soluble TNF- α , has lytic activity and may also have an important role in intercellular communication (15 - 17).

Two distinct receptor types have been identified that specifically bind TNF- α and TNF- β . Virtually all cell types studied show the presence of one or both of these receptor types. One type, TNFR-II (or Type A, or Type α , or 75 kDa or utr antigen), is a transmembrane glycoprotein with an apparent molecular weight of 75 kDa (18). The other type, TNFR-I (or Type B, or Type β , or 55 kDa or htr antigen), is a transmembrane glycoprotein with an apparent molecular weight of 55 kDa (19, 20). The two receptor types are distinct immunologically, but show similarities to each other and to the NGF receptor in the pattern of cysteine residue locations in four domains in their extracellular portions (5, 18). The intracellular domains of the two TNF receptor types are apparently unrelated, suggesting that the two receptor types employ different signal transduction pathways (18). Each receptor type can bind TNF- α or TNF- β with high affinity and there is no evidence that interaction between the two receptor types is necessary for signal transduction (20 - 22). Soluble forms of both types of receptors have been found in human serum and urine (23 - 25). These soluble receptors are capable of neutralizing the biological activities of both TNF- α and TNF- β and may serve to modulate and localize the activities of the TNFs or may serve as a reservoir for the controlled release of the TNFs.

The two TNFs are extremely pleiotropic factors. That they are capable of producing such a wide variety of effects is attributable to the ubiquity of their receptors, to their ability to activate multiple signal transduction pathways, and to their ability to induce or suppress the expression of a vast number of genes, including those for growth factors and cytokines, transcription factors, receptors, inflammatory mediators and acute phase proteins, *etc.* (5, 26). TNFs play a critical role in normal host resistance to infections and to the growth of malignant tumors, serving as immunostimulants and as mediators of the inflammatory response. Many of the actions produced by the TNFs are functionally similar to the effects produced by IL-1.

On the other hand, over-production of TNF has been implicated as playing a role in a number of pathological conditions, including cachexia (progressive wasting) (2, 27), septic shock following infection with Gram-negative bacteria (28), autoimmune disorders (29), and meningococcal septicemia (30). Two studies have found elevated levels of TNF- α in the cerebrospinal fluid (CSF) of multiple sclerosis (MS) patients, particularly those with active rather than stable disease (31, 32). TNF- α was also detected histologically in MS lesions (33). TNF levels in serum was a less useful indicator of patient status than levels in CSF.

Current bioassays used for the detection of TNF- α are usually based on the cytolytic effects of TNF- α on responsive cell lines, such as L929. These assays are tedious and are not specific for human TNF- α . The Quantikine HS TNF- α Immunoassay is an 18.5 - 24.5 hour (with an overnight incubation step) solid phase ELISA designed to measure TNF- α in serum, plasma, and urine. 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 on naturally occurring TNF- α samples showed linear curves that were parallel to the standard curves obtained using the *E. coli*-expressed Quantikine HS kit standards. These results indicate that the Quantikine HS Immunoassay kit can be used to determine relative mass values for natural 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.

- 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.

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. 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.

AMPLIFICATION SYSTEM

The Quantikine HS Immunoassay kit uses an amplification system in which the alkaline phosphatase reaction provides a cofactor that activates a redox cycle leading to the formation of a colored product (34 - 36). In this amplification system, alkaline phosphatase dephosphorylates the reduced form of nicotinamide adenine dinucleotide phosphate, NADPH (Substrate), to reduced nicotinamide adenine dinucleotide, NADH. The NADH subsequently serves as a specific cofactor that activates a redox cycle driven by the secondary enzyme system consisting of alcohol dehydrogenase and diaphorase (Amplifier). In the reaction catalyzed by diaphorase, NADH reduces a tetrazolium salt (INT-violet or iodionitrotetrazolium violet) to produce an intensely colored formazan dye and NAD⁺. NAD⁺ in turn is reduced by ethanol, in an alcohol dehydrogenase-catalyzed reaction, to regenerate NADH which can then re-enter the redox cycle. The rate of reduction of the tetrazolium salt and thus the amount of colored product formed are directly proportional to the amount of TNF- α bound in the initial step.

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.
- It is important that the Calibrator Diluent selected for the standard curve be consistent with the samples being assayed.
- Although this kit has been designed to eliminate serum matrix problems, some serum samples may exist that give falsely elevated values when assayed neat. If either serum or urine 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.

REAGENTS

TNF- α Microplate (Part 890239) - 96 well polystyrene microplate (12 strips of 8 wells) coated with a murine monoclonal antibody against TNF- α .

TNF- α Conjugate (Part 890240) - 21 mL of polyclonal antibody against TNF- α , conjugated to alkaline phosphatase, with preservative.

TNF- α Standard (Part 890146) - 3 vials (32 pg/vial) of recombinant human TNF- α in a buffered protein base with preservative, lyophilized.

Assay Diluent HD1-11 (Part 895130) - 6 mL of a buffered protein base with preservative.

Calibrator Diluent HD5N (Part 895147) - 21 mL of a buffered protein base with preservative. *For urine samples.*

Calibrator Diluent HD6J (Part 895070) - 21 mL of animal serum with preservative. *For serum/plasma samples.*

Wash Buffer Concentrate (Part 895073) - 100 mL of a 5-fold concentrated solution of buffered surfactant with preservative.

Substrate (Part 895077) - Lyophilized NADPH with stabilizers.

Substrate Diluent (Part 895078) - 7 mL of buffered solution with stabilizers.

Amplifier (Part 895075) - Lyophilized amplifier enzymes with stabilizers.

Amplifier Diluent (Part 895076) - 7 mL of buffered solution containing ethanol and INT-violet with stabilizers.

Stop Solution (Part 895074) - 6 mL of 2N sulfuric acid.

Plate Covers - 8 Adhesive strips.

STORAGE

Unopened Kit	Store at 2 - 8°C. Do not use past kit expiration date.	
Opened/ Reconstituted Reagents	Diluted Wash Buffer	Store for up to 1 month at 2 - 8° C.*
	Stop Solution	
	Assay Diluent HD1-11	
	Calibrator Diluent HD5N	
	Calibrator Diluent HD6J	
	Conjugate	
	Standard	Store for up to 4 hours at 2 - 8° C. Do not use standards that have been reconstituted for more than 4 hours.
	Substrate Solution	Store for up to 1 month at \leq - 20° C*. Avoid repeated freeze-thaw cycles.
	Amplifier Solution	
	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 490 nm, preferably with dual wavelength correction (correction wavelength set at 650 nm or 690 nm).
- Pipettes: 50 μ L and 200 μ L for running the assay; 1 mL and 5 mL for reagent preparation.
- Deionized or distilled water.
- Multi-channel pipette, squirt bottle or manifold dispenser.
- Graduated cylinders: 100 mL and 500 mL for preparation of Wash Buffer.
- 2 - 8° C incubator or refrigerator.

PRECAUTIONS

Some components of 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.

The Stop Solution provided with this kit is an acid solution. Wear eye, hand, face, and clothing protection when using this material.

This kit contains Thimerosal, a mercury containing compound. The total amount of mercury in this kit is 12.3 mg.

SAMPLE COLLECTION AND STORAGE

Serum - Use a serum separator tube (SST) and allow samples to clot for 30 minutes before centrifugation for 10 minutes at approximately 1000 x g. Remove serum and assay immediately or aliquot and store samples at $\leq -20^{\circ}\text{C}$. Avoid repeated freeze-thaw cycles.

Plasma - Collect plasma using EDTA or citrate as an anticoagulant. Centrifuge within 30 minutes of collection. Assay immediately or aliquot and store samples at $\leq -20^{\circ}\text{C}$. Avoid repeated freeze-thaw cycles. **Note:** *Heparinized plasma is not recommended 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 and assay immediately or aliquot and store at $\leq -20^{\circ}\text{C}$. Avoid repeated freeze-thaw cycles. For more information on collecting and handling urine specimens, refer to the National Committee for Clinical Laboratory Standards (NCCLS) publication GP16-T.

REAGENT PREPARATION

Bring all reagents to room temperature before use.

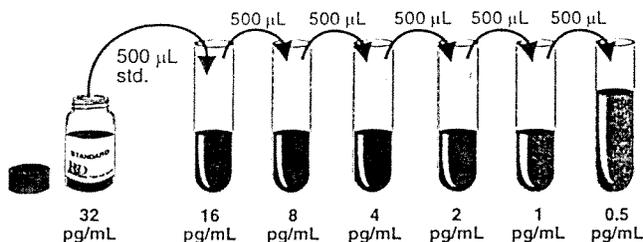
Wash Buffer - If crystals have formed in the concentrate, warm to room temperature and mix gently until the crystals have completely dissolved. Dilute 100 mL of Wash Buffer Concentrate into deionized or distilled water to prepare 500 mL of Wash Buffer.

Substrate Solution - Reconstitute the lyophilized Substrate in 6 mL of Substrate Diluent at least 10 minutes before use. Mix thoroughly. **Discard the rubber stopper after reconstitution.** Avoid contamination.

Amplifier Solution - Reconstitute the lyophilized Amplifier in 6 mL of Amplifier Diluent at least 10 minutes before use. Mix thoroughly. **Discard the rubber stopper after reconstitution.**

TNF- α Standard - Reconstitute the TNF- α Standard with 1 mL of Calibrator Diluent HD5N (for urine samples) or Calibrator Diluent HD6J (for serum/plasma samples). 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.

Pipette 500 μL of the appropriate Calibrator Diluent 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). The appropriate Calibrator Diluent 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 samples and standards 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, reseal.
3. Add 50 μL of Assay Diluent HD1-11 to each well.
4. Add ¹⁰⁰200 μL of Standard or sample per well. Cover with the adhesive strip provided. Incubate for 14 - 20 hours at 2 - 8 $^{\circ}\text{C}$.
5. Wash

Notes on washing

- a. Excessive drying of the wells can lead to poor assay performance and imprecision. Subsequent reagents should be added immediately after washing the plate, and the wells not allowed to dry completely. Also avoid prolonged exposure of the wells to vacuum aspiration apparatus.
- b. After removal of the adhesive strip prior to washing, the strip can be adhered to the underside of the wells to avoid the possibility of wells becoming dislodged during the decanting and rapping operations.
- c. Inclusion of a 30 second soak between each addition of Wash Buffer and decanting the plate contents will improve the precision of the assay.

Wash Procedure

- i. Remove liquid from the wells by inverting the plate and decanting the contents.
 - ii. Remove excess liquid by grasping the plate firmly and smartly rapping the plate inverted on a clean paper towel at least 5 times.
 - iii. Fill each well with 400 μL of Wash Buffer using a squirt bottle, multi-channel pipette or manifold dispenser.
 - iv. Remove liquid from the wells by inverting the plate and decanting the contents.
 - v. Repeat steps ii, iii, and iv 3 times for a total of 4 washes. After the last wash, smartly rap the plate inverted on a clean paper towel at least 10 times to remove excess Wash Buffer.
6. Add 200 μL of TNF- α Conjugate to each well. Cover with a new adhesive strip. Incubate for 3 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 60 minutes 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.

EM - 8 channel washer; reverse plate position w/ @ wash
blanks not in G, H 11, 12; EM's D 9, 10
or E 9, 10

ASSAY PROCEDURE SUMMARY

1. Prepare all reagents and standards as instructed.



2. Add 50 μ L Assay Diluent HD1-11 to each well.



3. Add ¹⁰⁰~~200~~ μ L Standard or sample to each well.
Incubate 14-20 hrs. at 2 - 8° C.



4. Wash ~~4~~ times. **4**



5. Add 200 μ L Conjugate to each well.
Incubate 3 hrs. RT



6. Wash ~~4~~ times. **4**



7. Add 50 μ L Substrate Solution to each well.
Incubate 1 hr. RT



8. Add 50 μ L Amplifier Solution to each well.
Incubate 30 min. RT



9. Add 50 μ L Stop Solution to each well.
Read at 490 nm within 30 min.
 λ correction 650 or 690 nm

CALCULATION OF RESULTS

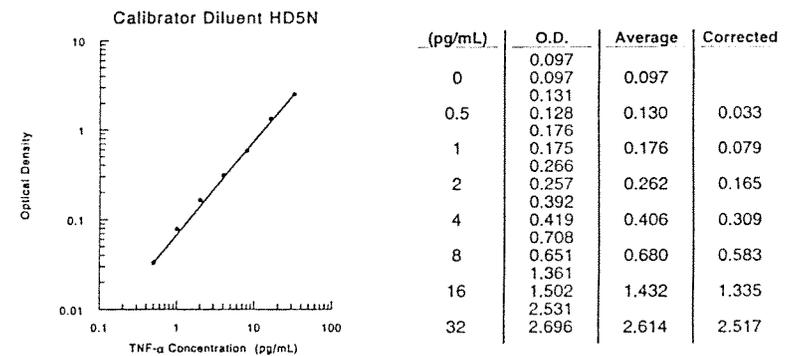
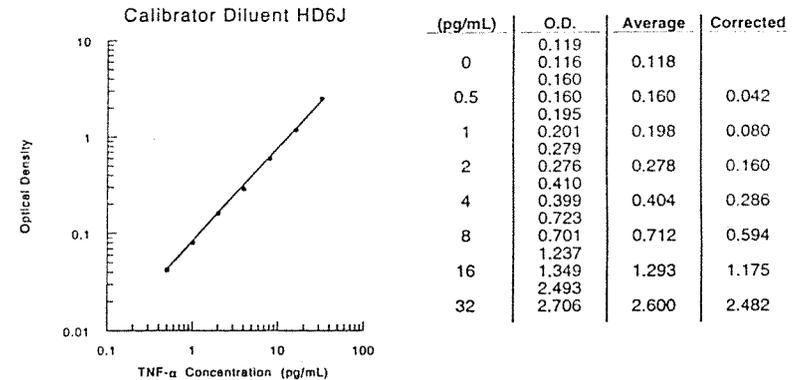
Average the duplicate readings for each standard and sample and subtract the average zero standard optical density.

Plot the optical density for the standards versus the concentration of the standards and draw the best curve. The data can be linearized by using log/log paper and regression analysis may be applied to the log transformation.

To determine the TNF- α concentration of each sample, first find the absorbance value on the y-axis and extend a horizontal line to the standard curve. At the point of intersection, extend a vertical line to the x-axis and read the corresponding TNF- α concentration. If samples have been diluted, the concentration read from the standard curve must be multiplied by the dilution factor.

TYPICAL STANDARD CURVES

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



TECHNICAL HINTS

- 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.
- 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.
- To ensure accurate results, proper adhesion of plate sealers during incubation steps is necessary.

PRECISION

Intra-assay Precision (Precision within an assay)

Three samples of known concentration were assayed in replicates of twenty to assess intra-assay precision.

Inter-assay Precision (Precision between assays)

Three samples of known concentration were evaluated 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)	0.81	5.91	10.68	0.84	6.37	11.27
Standard deviation	0.12	0.85	0.93	0.19	1.19	1.81
CV (%)	14.3	14.3	8.7	22.6	18.7	16.1

Urine Assay

Sample	Intra-Assay Precision			Inter-Assay Precision		
	1	2	3	1	2	3
n	20	20	20	20	20	20
Mean (pg/mL)	1.01	5.54	10.06	1.14	7.02	12.13
Standard deviation	0.18	0.10	1.83	0.17	0.97	1.41
CV (%)	17.6	18.6	18.2	14.9	13.8	11.6

RECOVERY

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

Sample Type	Average % Recovery	Range
Serum	95	81 - 105%
EDTA plasma	96	86 - 105%
Citrate plasma	98	85 - 106%
Urine	102	94 - 112%

LINEARITY

To assess linearity of the assay, the following biological samples containing or spiked with high concentrations of TNF- α , were diluted with Calibrator Diluent HD5N or HD6J and then assayed.

		Serum	EDTA plasma	Citrate plasma	Urine
1:2	Average % Recovery	101	102	98	100
	Range (%)	96-110	90-111	86-107	89-106
1:4	Average % Recovery	101	98	91	97
	Range (%)	95-115	91-111	80-103	89-106
1:8	Average % Recovery	105	102	91	99
	Range (%)	95-111	94-118	80-103	95-102

SENSITIVITY

The minimum detectable dose of TNF- α was typically less than 0.18 pg/mL.

The minimum detectable dose was determined by adding two standard deviations to the mean optical density value of 20 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 TNF- α International Reference Standard 87/650, which is intended as a potency standard, was evaluated in this kit. This standard is an *E. coli*-expressed TNF- α .

The dose response curve of this First International Standard parallels the Quantikine HS standard curve. To convert sample values obtained with the Quantikine HS TNF- α kit to equivalent NIBSC 87/650 nominally assigned mass values when using either Calibrator Diluent HD6J or Calibrator Diluent HD5N, use the equation below.

NIBSC/WHO (87/650) equivalent value (IU/mL) = 0.03 x Quantikine HS TNF- α value, (pg/mL)

SAMPLE VALUES

Serum, plasma, and urine samples were evaluated in this assay.

Sample Type	Range (pg/mL)	% Detectable	Mean of Detectable (pg/mL)
Serum (n=75)	ND - 3.62	99	1.25
Plasma EDTA (n=75)	ND - 4.12	88	1.42
Plasma citrate (n=43)	ND - 3.78	65	1.67
Urine (n=51)	ND - 1.94	43	0.82

ND = non-detectable (< 0.5 pg/mL)

SPECIFICITY

This assay recognizes both natural and recombinant human TNF- α . Preparations of the following factors at 10 ng/mL in a mid-range rhTNF- α control prepared in Calibrator Diluent HD6J and Calibrator Diluent HD5N were assayed. No significant cross-reactivity or interference was observed.

Factors related to or associated with TNF- α :

rhsTNF RI rhsTNF RII rhTNF- β rmTNF- α

Other factors:

Recombinant human	IL-11	LIF	TGF- β 3	SCF
IL-1 α	ANG	M-CSF	Recombinant mouse:	Other:
IL-1 β	CNTF	MCP-1	IL-1 α	mEGF
IL-1 ra	β -ECGF	MIP-1 α	IL-1 β	hPDGF
IL-2	EGF	MIP-1 β	IL-3	pPDGF
IL-3	Epo	OSM	IL-4	hTGF- β 1
IL-4	FGF acidic	PDGF-AA	IL-5	pTGF- β 1
IL-5	FGF basic	PDGF-AB	IL-6	pTGF- β 1.2
IL-6	FGF-4	PDGF-BB	IL-7	pTGF- β 2
IL-6 sR	G-CSF	RANTES	IL-9	rcTGF- β 3
IL-7	GM-CSF	SCF	IL-12	raTGF- β 5
IL-8	GRO α	SLPI	GM-CSF	
IL-9	IFN- γ	TGF- α	MIP-1 α	
IL-10	IGF-I	TGF- β 1	MIP-1 β	
	IGF-II	TGF- β 2		

No cross-reactivity or interference was observed with recombinant canine TNF- α at concentrations less than 10 ng/mL.

APPLICABLE PATENTS

These products are covered by the following patents:

Substrate (Part 895077) - Lyophilized NADPH with stabilizers.

Substrate Diluent (Part 895078) - 7 mL of buffered solution with stabilizers.

Amplifier (Part 895075) - Lyophilized amplifier enzymes with stabilizers.

Amplifier Diluent (Part 895076) - 7 mL of buffered solution containing ethanol and INT-violet with stabilizers.

US:
4,446,231
4,595,655
4,598,042

EUROPE:
60,123
27,036

CANADA:
1,170,179

AUSTRALIA:
544,496

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