

Quantikine[®] HS

Human TNF- α /TNFSF1A Immunoassay

Catalog Number HSTA00C

For the quantitative determination of human tumor necrosis factor alpha (TNF- α) 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 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, TNF RII (or Type A, Type α , 75 kDa or utr antigen), is a transmembrane glycoprotein with an apparent molecular weight of 75 kDa (18). The other type, TNF RI (or Type B, Type β , 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).

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 a 6.5 hour solid phase ELISA designed to measure 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 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 TNF- α 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 iodinitrotetrazolium 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.
- Do not mix or substitute reagents with those from other sources or lots.
- Although this kit has been designed to eliminate matrix problems, some samples may exist 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 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 890953) - 96 well polystyrene microplate (12 strips of 8 wells) coated with a murine monoclonal antibody against TNF- α .

TNF- α Conjugate (Part 890954) - 21 mL of polyclonal antibody against TNF- α , conjugated to alkaline phosphatase, with preservative.

TNF- α Standard (Part 890955) - 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. Contains a precipitate.

Calibrator Diluent RD6-13 (Part 895491) - 21 mL of a buffered protein base with preservative.

Wash Buffer Concentrate (Part 895188) - 100 mL of a 10-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 INT-violet with stabilizers.

Stop Solution (Part 895032) - 6 mL of 2 N 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 RD6-13	
	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 ≤ - 20° C* in a manual defrost freezer. 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 set at 650 nm or 690 nm.
- Pipettes and pipette tips.
- Deionized or distilled water.
- Multi-channel pipette, squirt bottle, manifold dispenser or automated microplate washer.
- 1000 mL graduated cylinder for preparation of Wash Buffer.
- Human TNF- α Controls (optional; available from R&D Systems).

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 11.05 mg.

SAMPLE COLLECTION AND STORAGE

Serum - Use a serum separator tube (SST) and allow samples to clot for 30 minutes before centrifugation for 15 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.

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

REAGENT PREPARATION

Bring all reagents to room temperature before use.

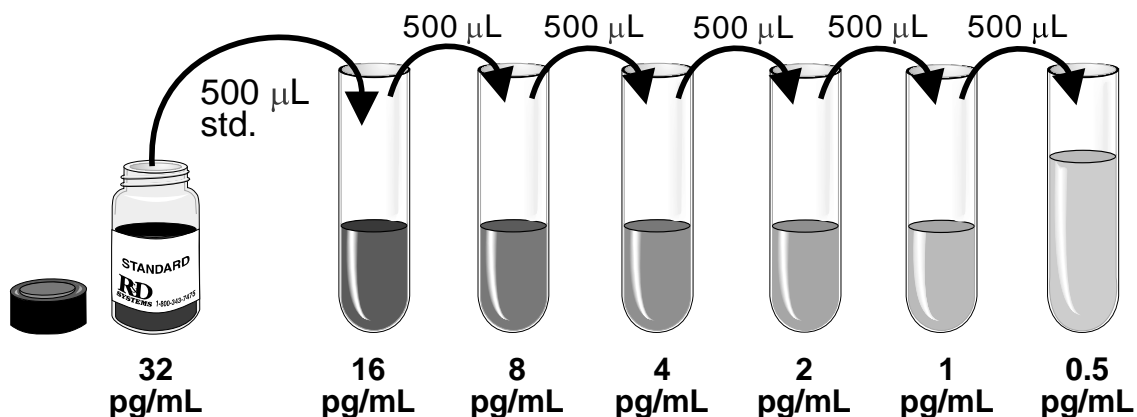
Wash Buffer - If crystals have formed in the concentrate, warm to room temperature and mix gently until the crystals have completely dissolved. Dilute 100 mL of Wash Buffer Concentrate into deionized or distilled water to prepare 1000 mL of Wash Buffer.

Substrate Solution - Reconstitute the lyophilized Substrate with 6.0 mL of Substrate Diluent at least 10 minutes before use. Mix thoroughly. **Discard the rubber stopper after reconstitution.** Avoid contamination.

Amplifier Solution - Reconstitute the lyophilized Amplifier with 6.0 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 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.

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).



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, reseal.
3. Add 50 μL of Assay Diluent HD1-11 to each well. Assay Diluent HD1-11 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.

5. Wash

Notes on washing

- *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.*
- *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.*
- *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

- 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, multi-channel pipette, 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 TNF- α HS Conjugate to each well. 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.

ASSAY PROCEDURE SUMMARY

1. Prepare all reagents and standards as instructed.



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



3. Add 200 μL Standard or sample to each well.
Incubate 3 hrs. at RT



4. Wash 6 times.



5. Add 200 μL Conjugate to each well.
Incubate 2 hrs. RT



6. Wash 6 times.



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



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



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

CALCULATION OF RESULTS

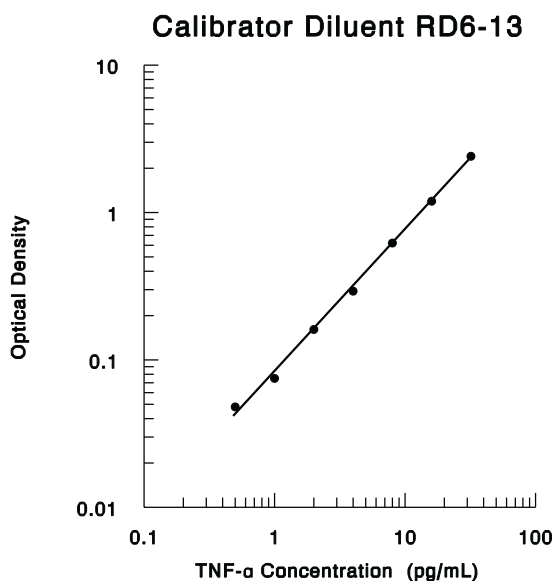
Average the duplicate readings for each standard and sample and subtract the average zero standard optical density.

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 CURVE

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	0.104 0.109 0.154	0.107	—
0.5	0.155 0.180	0.155	0.048
1	0.184 0.266	0.182	0.075
2	0.269 0.402	0.268	0.161
4	0.397 0.718	0.400	0.293
8	0.737 1.315	0.728	0.621
16	1.287 2.611	1.301	1.194
32	2.421	2.516	2.409

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.
- 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.

PRECISION

Intra-assay Precision (Precision within an assay)

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

Inter-assay Precision (Precision between assays)

Three samples of known concentration were evaluated in forty separate assays to assess inter-assay precision.

Sample	Intra-Assay Precision			Inter-Assay Precision		
	1	2	3	1	2	3
n	20	20	20	40	40	40
Mean (pg/mL)	2.6	7.2	14.0	2.4	6.7	13.5
Standard deviation	0.23	0.43	0.74	0.41	0.85	1.46
CV (%)	8.8	5.9	5.3	16.7	12.6	10.8

RECOVERY

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

Sample Type	Average % Recovery	Range
Serum	100	91 - 108%
EDTA plasma	97	88 - 106%
Citrate plasma	100	90 - 115%
Heparin plasma	106	90 - 115%

LINEARITY

To assess linearity of the assay, samples were spiked with high concentrations of TNF- α in various matrices and diluted with Calibrator Diluent RD6-13 to produce samples with values within the dynamic range of the assay.

		Serum (n=10)	EDTA plasma (n=10)	Citrate plasma (n=10)	Heparin plasma (n=5)
1:2	Average % Recovery	101	100	101	102
	Range (%)	91 - 105	95 - 107	98 - 105	94 - 111
1:4	Average % Recovery	99	96	93	98
	Range (%)	93 - 105	89 - 101	91 - 99	91 - 105
1:8	Average % Recovery	97	100	99	97
	Range (%)	89 - 102	95 - 109	93 - 109	88 - 104
1:16	Average % Recovery	91	105	101	98
	Range (%)	86 - 101	93 - 115	97 - 107	87 - 105

SENSITIVITY

Forty assays were evaluated and the minimum detectable dose (MDD) of TNF- α ranged from 0.06 pg/mL to 0.32 pg/mL. The mean MDD was 0.12 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 TNF- α First International Reference Standard 87/650, 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 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, 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 and plasma samples were evaluated in this assay.

Sample Type	Range (pg/mL)	% Detectable	Mean of Detectable (pg/mL)
Serum (n = 32)	ND - 4.71	97	2.07
EDTA plasma (n = 35)	ND - 4.22	97	1.98
Citrate plasma (n = 21)	0.71 - 2.07	100	1.22
Heparin plasma (n = 30)	ND - 3.86	97	1.99

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

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

SPECIFICITY

This assay recognizes recombinant and natural human TNF- α . The factors listed below were prepared at 50 ng/mL in Calibrator Diluent RD6-13 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.

Factors related to or associated with TNF- α :

rhsTNF RI rhsTNF RII rhTNF- β rmTNF- α rrTNF- α rpTNF- α

Other factors:

Recombinant human:	IL-4	SLPI	IL-9	IL-2
ANG	IL-5	SMDF	IL-10	IL-4
ANG-2	IL-6	Tpo	IL-11	IL-6
AR	IL-7	TRAIL	IL-12	IL-10
BDNF	IL-8	TRANCE	IL-12 p40	Leptin
CD4	IL-10	Recombinant mouse:	IL-13	MK
CD40	IL-11	CT-1	IL-17	Recombinant porcine:
CD40 Ligand	IL-12	CTLA-4	Leptin	IL-1 α
CNTF	IL-12 p40	Fas	LIF	IL-1 β
CT-1	IL-13	Fas Ligand	OPG	IL-2
CTLA-4	IL-15	GITR Ligand	OPN	IL-4
Epo	IL-16	IFN- γ	OSM	IL-6
Fas	IL-17	IL-1 α	SCF	IL-8
GDNF	Leptin	IL-1 β	Tpo	IL-10
GITR	LIF	IL-1ra	TRANCE	
IFN- γ	MIF	IL-2	Recombinant rat:	
IL-1 α	NT-3	IL-3	CNTF	
IL-1 β	NT-4	IL-4	GDNF	
IL-1ra	OPG	IL-5	IFN- γ	
IL-2	OSM	IL-6	IL-1 α	
IL-3	PTN	IL-7	IL-1 β	
	SCF			

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 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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