

Human TNF- β ELISA Kit

For the Quantitative Determination of Human Tumour Necrosis
Factor Beta (TNF- β) Concentrations in Serum, Plasma, and Cell
Culture Supernatant

Catalogue Number: EL10047

96 tests

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

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INTENDED USE

This Human TNF- β ELISA kit is to be used for the *in vitro* quantitative determination of human Tumour Necrosis Factor Beta (TNF- β) concentrations in serum, plasma, and cell culture supernatant. This kit is intended for LABORATORY RESEARCH USE ONLY and is not for use in diagnostic or therapeutic procedures.

INTRODUCTION

TNF-beta (TNF- β) is a protein of 171 amino acids in length and N-glycosylated at position 62. Some cell lines secrete varying glycosylated forms of the factor that may differ also in their biological activities. The protein does not contain disulfide bonds and forms heteromers with LT-beta (Lymphotoxin-beta) that anchors the complexes in the membrane. TNF- β and TNF- α show approximately 30 percent sequence homology and bind to the same receptor, and murine and human TNF- β are highly homologous at 74 percent.

The biological functions of human TNF- β (hTNF- β) include promoting lymphoid development, enhancing normal host resistance to infection, and inhibiting growth of malignant tumors. Elevated hTNF- β levels are usually related to Crohn's Disease, multiple sclerosis, juvenile rheumatoid adenoleucodystrophy, hypercalcemia of adult T cell leukemia, arthritis, bulbous pemphigoid, non-Hodgkin's lymphoma, and pregnancy. TNF- β is cytolytic or cytostatic for many tumor cells and acts as a mitogen for B-lymphocytes. TNF- β is also a chemo-attractant for neutrophils, increases phagocytosis, and increases adhesion to the endothelium. TNF- β promotes the proliferation of fibroblasts and is likely involved in the wound healing processes *in vivo*. TNF- β not only induces the synthesis of GM-CSF, G-CSF, IL-1, but also acts as an anti-angiogenesis factor. Hemorrhagic necrosis of tumors induced by TNF- β *in vivo* is likely the result of an inhibition of the growth of endothelial cells.

The TNF- β gene has a length of approximately 3 kb and contains four exons. The gene maps to human chromosome 6p23-6q12, which is approximately 1.2 kb apart from the TNF- α gene. The results obtained from the study of the TNF- β gene polymorphisms suggest that the gene may modify individual susceptibility to breast cancer in women.

This hTNF- β ELISA Kit is a ready-to-use 3-hour solid phase immunoassay readily capable of measuring hTNF- β in the range of 0 to 4000 pg/mL in cell culture supernatant, serum and plasma. However, data collection for proliferation testing for hTNF- β detection will require additional days for completion. The assay has shown a specific reaction with hTNF- β and no cross-reactivity with various other cytokine superfamily proteins.

PRINCIPLE OF THE ASSAY

This TNF- β enzyme linked immunosorbent assay (ELISA) applies a technique called a quantitative sandwich immunoassay. The microtiter plate provided in this kit has been pre-coated with a monoclonal antibody specific to TNF- β . Standards or samples are then added to the appropriate microtiter plate wells and incubated. After washing to remove unbound TNF- β and other components of the sample, biotin-conjugated polyclonal antibody specific to TNF- β is added and incubated. If present, TNF- β will bind and become immobilized by the antibody pre-coated on the wells and then become "sandwiched" by the biotin conjugate. In order to quantitatively determine the amount of TNF- β present in the sample, Avidin conjugated to Horseradish Peroxidase (HRP) is added to each microplate well and incubated. Avidin is a tetramer containing four identical subunits, each having a high affinity-binding site for biotin. The wells are thoroughly washed to remove all unbound HRP-conjugated Avidin and a TMB (3,3',5,5' tetramethyl-benzidine) substrate solution is added to each well. The enzyme (HRP) and substrate are allowed to react over a short incubation period. Only those wells that contain TNF- β , biotin-conjugated antibody and enzyme-conjugated Avidin will exhibit a change in colour. The enzyme-substrate reaction is terminated by the addition of a sulphuric acid solution and the colour change is measured spectrophotometrically at a wavelength of 450 nm \pm 2 nm.

This kit includes two calibration diluents (Calibrator Diluent I for serum/plasma testing and Calibrator Diluent II for cell culture supernatant testing) in order to measure the concentration of TNF- β in the samples. According to the testing system, the standard provided is diluted (2-fold) with the appropriate Calibrator Diluent and assayed at the same time as the samples. This allows the operator to produce a standard curve of Optical Density (O.D) versus TNF- β concentration (pg/mL). The concentration of TNF- β in the samples is then determined by comparing the O.D. of the samples to the standard curve.

LIMITATIONS OF APPLICATION

- The Human TNF- β ELISA kit is not for use in clinical diagnostic procedures, and for laboratory use only.
- Although all manufacturing precautions have been exercised to ensure that this product will be suitable for use with all validated sample types as designated in the product insert, the possibility of interference cannot be excluded due to the variety of proteins that may exist within the sample.
- The Calibrator Diluent selected for the standard curve should be consistent with the assay samples. If the values generated by the samples are greater than the uppermost standard, the samples dilution should be adjusted with the appropriate Calibrator Diluent and the assay should be repeated.

REAGENTS PROVIDED

All reagents provided are stored at 4°C. Refer to the expiration date on the label.

		96 tests
1.	TNF-β MICROTITER PLATE (Part 47009) _____ Pre-coated with murine anti-human TNF-β monoclonal antibody.	96 wells
2.	BIOTIN CONJUGATE (Part 47010) _____ Anti-human TNF-β polyclonal antibody conjugated to Biotin.	11 mL
3.	AVIDIN CONJUGATE (Part 47011) _____ Avidin conjugated to horseradish peroxidase.	14 mL
4.	TNF-β STANDARD (Part 47012) _____ Recombinant human TNF-β (8.0 ng/vial) in a buffered protein base with preservative, lyophilized.	2 vials
5.	SERUM ASSAY BUFFER (Part 47013) _____ Designed for TNF-β serum sample assay	6 mL
6.	CALIBRATOR DILUENT I (Part 30003) _____ Animal protein with buffer and preservative. <i>For serum/plasma testing.</i>	22 mL
7.	CALIBRATOR DILUENT II (Part 30004) _____ Cell culture medium with animal protein and preservative. <i>For cell culture supernatant testing.</i>	22 mL
8.	WASH BUFFER (20X) (Part 30005) _____ 20-fold concentrated solution of buffered surfactant.	60 mL
9.	SUBSTRATE A (Part 30006) _____ Buffered solution with H ₂ O ₂	10 mL
10.	SUBSTRATE B (Part 30007) _____ Buffered solution with TMB.	10 mL
11.	STOP SOLUTION (Part 30008) _____ 2N Sulphuric Acid (H ₂ SO ₄). Caution: Caustic Material!	14 mL

MATERIALS REQUIRED BUT NOT SUPPLIED

1. Single or multi-channel precision pipettes with disposable tips: 10-100 μ L and 50-200 μ L for running the assay.
2. Pipettes: 1 mL, 5 mL 10 mL, and 25 mL for reagent preparation.
3. Multi-channel pipette reservoir or equivalent reagent container.
4. Test tubes and racks.
5. Polypropylene tubes or containers (25 mL).
6. Erlenmeyer flasks: 100 mL, 400 mL, 1 L and 2 L.
7. Microtiter plate reader (450 nm \pm 2nm)
8. Automatic microtiter plate washer or squirt bottle.
9. Sodium hypochlorite solution, 5.25% (household liquid bleach).
10. Deionized or distilled water.
11. Plastic plate cover.
12. Disposable gloves.
13. Absorbent paper.

PRECAUTIONS

1. Do not substitute reagents from one kit lot to another. Standard, conjugate and microtiter plates are matched for optimal performance. Use only the reagents supplied by manufacturer.
2. Allow kit reagents and materials to reach room temperature (20-25°C) before use. Do not use water baths to thaw samples or reagents.
3. Do not use kit components beyond their expiration date.
4. Use only deionized or distilled water to dilute reagents.
5. Do not remove microtiter plate from the storage bag until needed. Unused strips should be stored at 2-8°C in their pouch with the desiccant provided.
6. Use fresh disposable pipette tips for each transfer to avoid contamination.
7. Do not mix acid and sodium hypochlorite solutions.
8. Human serum and plasma should be handled as potentially hazardous and capable of transmitting disease. Disposable gloves must be worn during the assay procedure since no known test method can offer complete assurance that products derived from human blood will not transmit infectious agents. Therefore, all blood derivatives should be considered potentially infectious and good laboratory practices should be followed.
9. All samples should be disposed of in a manner that will inactivate human viruses.
Solid Wastes: Autoclave for 60 minutes at 121°C.
Liquid Wastes: Add sodium hypochlorite to a final concentration of 1.0%. The waste should be allowed to stand for a minimum of 30 minutes to inactivate the virus before disposal.
10. Substrate Solution is easily contaminated. If bluish prior to use, *do not use*.
11. Substrate B contains 20% acetone, keep this reagent away from sources of heat or flame.
12. If Wash Buffer (20X) is stored at a lower temperature (2-5°C), crystals may form which must be dissolved by warming to 37°C prior to use.

SAMPLE PREPARATION

1. COLLECTION, HANDLING, AND STORAGE

- a) **Cell Culture Supernatant:** Collect cell culture supernatant, Centrifuge to remove any visible pellets. Assay can be immediately conducted or samples can be aliquoted and store at $\leq -20^{\circ}\text{C}$. Avoid repeated freeze-thaw cycles.
- b) **Serum:** Use a serum separator tube (SST) and allow samples to clot for 30 minutes at room temperature. Centrifuge for 10 minutes at 1000 x g (4°C). Remove serum and assay (see activation procedure) immediately or aliquot and store at $\leq -20^{\circ}\text{C}$. Avoid repeated freeze-thaw cycles.
- c) **Plasma:** Collect plasma on ice using EDTA as an anticoagulant. Centrifuge at 1000 x g within 30 minutes of collection. An additional centrifugation step of the plasma at 10,000 x g for 30 minutes at $2-8^{\circ}\text{C}$ is recommended for complete platelet removal. Assay (see activation procedure) immediately or aliquot and store samples at $\leq -20^{\circ}\text{C}$. Avoid repeated freeze-thaw cycles.

PREPARATION OF REAGENTS

Remove all kit reagents from refrigerator and allow them to reach room temperature ($20-25^{\circ}\text{C}$). Prepare the following reagents as indicated below. Mix thoroughly by gently swirling before pipetting. Avoid foaming.

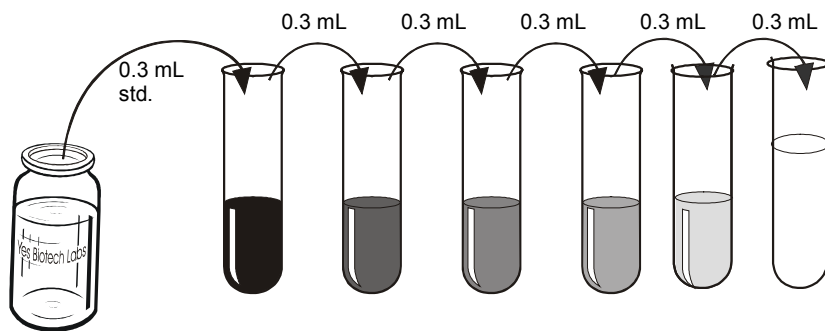
1. **Wash Buffer (1X):** Add 60 mL of Wash Buffer (20X) and dilute to a final volume of 1200 mL with distilled or deionized water. Mix thoroughly. If a smaller volume of Wash Buffer (1X) is desired, add 1 volume of Wash Buffer (20X) to 19 volumes of distilled or deionized water. Wash Buffer (1X) is stable for 1 month at $2-8^{\circ}\text{C}$. Mix well before use.
2. **Substrate Solution:** Substrate A and Substrate B should be mixed together in equal volumes up to 15 minutes before use. Refer to the table below for correct amounts of Substrate Solution to prepare.

Strips Used	Substrate A (mL)	Substrate B (mL)	Substrate Solution (mL)
2 strips (16 wells)	1.5	1.5	3.0
4 strips (32 wells)	3.0	3.0	6.0
6 strips (48 wells)	4.0	4.0	8.0
8 strips (64 wells)	5.0	5.0	10.0
10 strips (80 wells)	6.0	6.0	12.0
12 strips (96 wells)	7.0	7.0	14.0

3. **TNF-β Standard:**

a) Two vials of Standards are provided in this kit to allow both serum/plasma and cell culture supernatant testing. Reconstitute the TNF-β Standard with either 1.0 mL of Calibrator Diluent I (for serum/plasma testing) or 1.0 mL of Calibrator Diluent II (for cell culture supernate testing). This reconstitution produces a stock solution of 8000 pg/mL for testing. Allow solution to sit for at least 15 minutes with gentle agitation prior to making dilutions. Use within one hour of reconstituting. The TNF-β standard stock solution must be stored frozen (-20°C) immediately after use so that it can last for up to 30 days. Avoid freeze-thaw cycles. Aliquot if repeated use is expected.

4. **For Serum/Plasma Samples:** Use the 8000 pg/mL stock solution to produce a serial 2-fold dilution series within the range of this assay (0 pg/mL to 8000 pg/mL) as illustrated. Add 0.3 mL of the Calibrator Diluent I to each test tube. Each test tube transfer should be sure to mix contents thoroughly. The undiluted TNF-β Standard will serve as the **high standard (8000 pg/mL)** and the Calibrator Diluent will serve as the zero standard (0 pg/mL).



TNF-β Standard	4000	2000	1000	500	250	125
8000 pg/mL	pg/mL	pg/mL	pg/mL	pg/mL	pg/mL	pg/mL

5. **For Cell Culture Supernate Samples:** Use the 8000 pg/mL stock solution to produce a serial 2-fold dilution series within the range of this assay (0 pg/mL to 4000 pg/mL) as illustrated. Add 0.5 mL of the Calibrator Diluent II to each test tube. Each test tube transfer should be sure to mix contents thoroughly. The undiluted TNF-β Standard will serve as the **high standard (8000 pg/mL)** and the Calibrator Diluent will serve as the zero standard (0 pg/mL).

ASSAY PROCEDURE

1. Prepare Wash Buffer (1X) and TNF- β Standards and activated sample before starting assay procedure. *It is recommended that the table and diagram provided be used as a reference for adding Standards or Samples to the Microtiter Plate.*

Wells				Wells			
1A, 1B	Standard 1 - 0 pg/mL	(S1)		2A, 2B	Standard5 - 1000 pg/mL	(S5)	
1C, 1D	Standard 2 - 125 pg/mL	(S2)		2C, 2D	Standard6 - 2000 pg/mL	(S6)	
1E, 1F	Standard 3 - 250 pg/mL	(S3)		2E, 2F	Standard7- 4000 pg/mL	(S7)	
1G, 1H	Standard4 - 500 pg/mL	(S4)		2G, 2H	Standard8- 8000 pg/mL	(S8)	
				3A, 12H	TNF- β samples		

	1	2	3	4	5	6	7	8	9	10	11	12
A	S1	S5	1	5	9	13	17	21	25	29	33	37
B	S1	S5	1	5	9	13	17	21	25	29	33	37
C	S2	S6	2	6	10	14	18	22	26	30	34	38
D	S2	S6	2	6	10	14	18	22	26	30	34	38
E	S3	S7	3	7	11	15	19	23	27	31	35	39
F	S3	S7	3	7	11	15	19	23	27	31	35	39
G	S4	S8	4	8	12	16	20	24	28	32	36	40
H	S4	S8	4	8	12	16	20	24	28	32	36	40

2. **For Serum/Plasma Samples Only:** Add 50 μ L of Serum Assay Buffer to each well. Mix well before and during use. **For Cell Culture Supernatant Samples:** Start at Step 3.
3. Add 100 μ L of Standard or activated sample to the appropriate well of the antibody pre-coated Microtiter Plate. Cover and incubate for 1 hour at 37 °C.
4. Wash the Microtiter Plate using one of the specified methods indicated below:

Manual Washing: Remove incubation mixture by aspirating contents of the plate into a sink or proper waste container. Using a squirt bottle, fill each well completely with Wash Buffer (1X) then aspirate contents of the plate into a sink or proper waste container. Repeat this procedure one more time for a **total of five washes**. After final wash, invert plate, and blot dry by hitting plate onto absorbent paper or paper towels until no moisture appears. *Note:* Hold the sides of the plate frame firmly when washing the plate to assure that all strips remain securely in frame.

Automated Washing: Aspirate all wells, then wash plates **five times** using Wash Buffer (1X). Always adjust your washer to aspirate as much liquid as possible and

set fill volume at 350 μL /well/wash (range: 350-400 μL). After final wash, invert plate, and blot dry by hitting plate onto absorbent paper or paper towels until no moisture appears. *It is recommended that the washer be set for a soaking time of 10 seconds or shaking time of 5 seconds between washes.*

5. Dispense 100 μL biotin conjugate to each well. Mix well. Cover and incubate for 1 hour at 37 °C.
6. Repeat wash procedure as described in Step 4. Wash plate **five times**.
7. Dispense 100 μL avidin conjugate to each well. Mix well. Cover and incubate for 1 hour at 37 °C.
8. Repeat wash procedure as described in Step 4.
9. Prepare Substrate Solution no more than 15 minutes before end of second incubation (see Preparation of Reagents).
10. Add 100 μL Substrate Solution to each well. Cover and incubate for 15 minutes at 37 °C.
11. Add 100 μL Stop Solution to each well. Mix well.
12. Read the Optical Density (O.D.) at 450 nm using a microtiter plate reader set within 30 minutes.

CALCULATION OF RESULTS

The standard curve is used to determine the amount of TNF- β in an unknown sample. The standard curve is generated by plotting the average O.D. (450 nm) obtained for each of the standard concentrations on the vertical (Y) axis versus the corresponding TNF- β concentration (pg/mL) on the horizontal (X) axis.

1. First, calculate the mean O.D value for each standard and sample. All O.D. values are subtracted by the value of the zero-standard (0 pg/mL) or (S1) before result interpretation. Construct the standard curve using graph paper or statistical software.
2. To determine the amount of TNF- β in each sample, first locate the O.D. value on the Y-axis and extend a horizontal line to the standard curve. At the point of intersection, draw a vertical line to the X-axis and read the corresponding TNF- β concentration.

3. If samples generate values higher than the highest standard, dilute the samples with the appropriate Calibrator Diluent and repeat the assay. A suggested 10-fold dilution is 50 μ L sample + 450 μ L Calibrator Diluent I.

TYPICAL DATA

Results of a typical standard run of a TNF- β ELISA are shown below. Any variation in standard diluent, operator, pipetting and washing technique, incubation time or temperature, and kit age can cause variation in result. The following examples are for the purpose of illustration only, and should not be used to calculate unknowns. Each user should obtain their own standard curve

EXAMPLE ONE

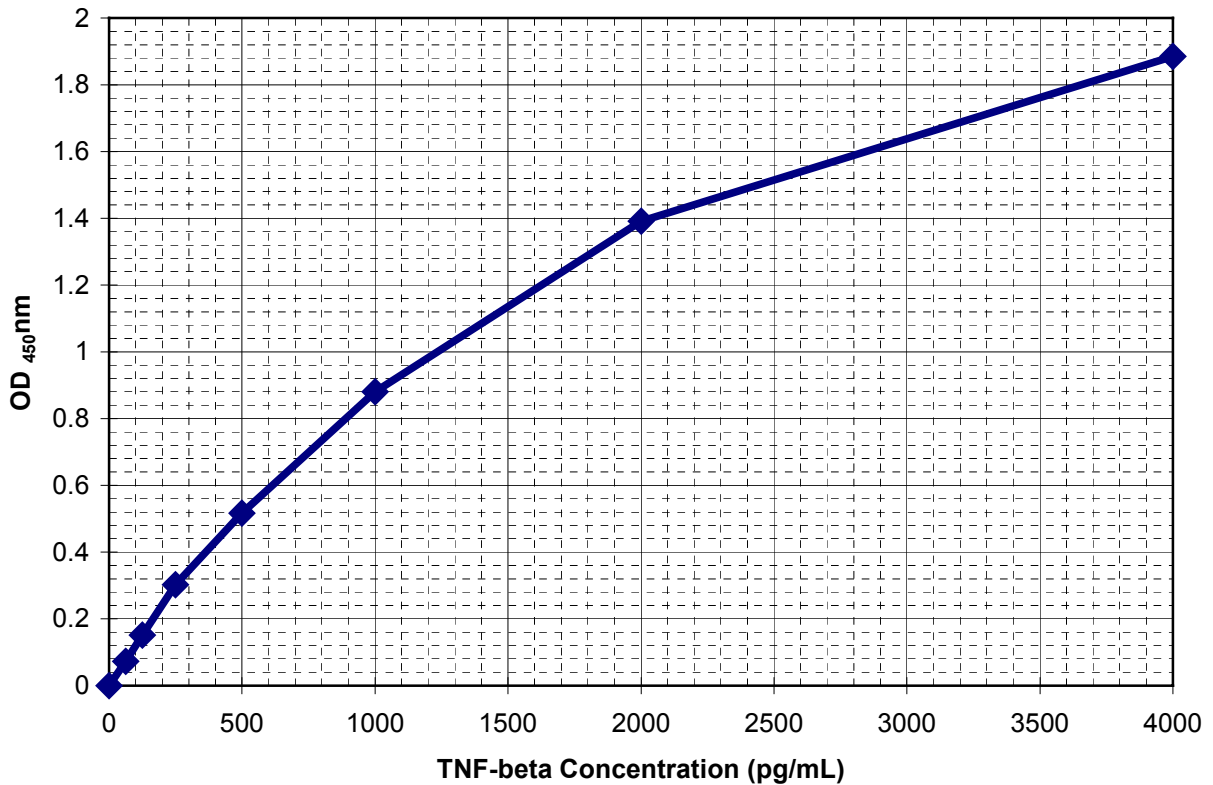
The following data was obtained for a standard curve using Calibrator Diluent I.

Standard (pg/mL)	O.D. (450 nm)	Zero Standard Subtracted (Std.)-(S1)
0	0.068	0
125	0.197	0.129
250	0.315	0.247
500	0.497	0.429
1000	0.800	0.732
2000	1.235	1.167
4000	1.787	1.719
8000	2.388	2.320

EXAMPLE TWO

The following data was obtained for a standard curve using Cell Culture Supernate samples with Calibrator Diluent II.

Standard (pg/mL)	O.D. (450 nm)	Mean	Zero Standard Subtracted (Std.)-(S1)
0	0.074, 0.072	0.073	0
125	0.144, 0.147	0.146	0.073
250	0.231, 0.217	0.224	0.151
500	0.381, 0.368	0.975	0.302
1000	0.590, 0.590	0.590	0.517
2000	0.961, 0.945	0.953	0.880
4000	1.480, 1.447	1.464	1.391
8000	1.968, 1.947	1.958	1.885



PERFORMANCE CHARACTERISTICS

1. INTRA-ASSAY PRECISION

To determine within-run precision, three different samples of known concentration were assayed by using 16 replicates in 1 assay.

Sample	<i>Calibrator Diluent I assay</i>			<i>Calibrator Diluent II assay</i>		
	1	2	3	1	2	3
n	16	16	16	16	16	16
Mean (pg/mL)	150	500	2000	150	500	2000
Standard Deviation (pg/mL)	12.0	25.1	90.7	7.8	14.5	48.0
Coefficient of Variation (%)	8.0	5.0	4.5	5.2	2.9	2.4

2. INTER-ASSAY PRECISION

To determine between-run precision, three different samples of known concentration were assayed by using replicates on 16 different assays.

Sample	Calibrator Diluent I assay			Calibrator Diluent II assay		
	1	2	3	1	2	3
n	16	16	16	16	16	16
Mean (pg/mL)	150	500	2000	150	500	2000
Standard Deviation (pg/mL)	13.9	30.1	100.9	8.8	20.9	70.0
Coefficient of Variation (%)	9.3	6.0	5.0	5.9	4.2	3.5

3. RECOVERY

By employing various samples, the recovery of TNF- β was evaluated with different amounts of TNF- β throughout the range of the assay.

Sample Type	Average % Recovery	Range
Serum	97	82-116%

4. SENSITIVITY

The minimum detectable quantities of human TNF- β as observed by the standard curve generated for both Calibrator Diluent I and Calibrator Diluent II are 22.0 pg/mL and 11.0 pg/mL respectively. The two standard deviations above the mean optical density of the 16 replicates of the zero standard were defined as the minimum detectable quantities.

5. SPECIFICITY

This assay can detect both natural and recombinant human TNF- β . The following factors were assayed at 10 ng/mL in Calibrator Diluent I for cross-reactivity. No significant cross-reactivity was observed.

Recombinant human: IL-1 α , IL-1 β , IL-2, IL-3, IL-4, IL-6, IL-8, TNF- α , GM-CSF

Other: bFGF acidic, hPDGF, pPDGF, hTGF- β 1, pTGF- β 1, pTGF- β 1.2, pTGF- β 2

6. CALIBRATION

This immunoassay is calibrated against NIBSC Standard (Reference preparation) Code No. 87/640.

7. SAMPLE VALUES

Serum - Fifty serum samples were tested in this assay and all had levels which fell below the lowest standard, 125 pg/mL.

REFERENCES

1. Aggarwal, B.B., Vilcek, J. (eds) (1992) Tumor Necrosis Factor: Structure, Function and Mechanism of Action, Marcel Dekker, New York.
2. Eck, M.J., *et al.* (1992) *J. Biol. Chem.* 267:2119.
3. Gray, P.W., *et al.* (1984) *Nature* 312:721.
4. Grom, A. A., (1996). *Arthritis Rheum.* 39, 1703-1710
5. Ishibashi, K., *et al.* (1991). *Blood* 77, 2451-2455
6. Jeffes, E. W., (1989). *Arthritis Rheum.* 32, 1148-1152
7. Laham, N., *et al.* (1997). *J. Reprod. Immuno.* 33, 53-69
8. Lee KM., *et al.* (2005) *Breast Cancer Res Treat.* 2005 Mar;90(2):149-55.
9. Noguchi, M., *et al.* (1998). *Gut* 43, 203-209
10. Ruddle, N.H. (1994) *The Cytokine Handbook*, A. Thomson ed., Academic Press, New York, p. 305
11. Schoenfeld, H.J. *et al.* (1991) *J. Biol. Chem.* 266:3863.
12. Selmaj, k., *et al.* (1997) *J. Clin. Invest.* 87, 949-954
13. Selmaj, K., *et al.* (1998) *Ann. Neurol.* 30, 694-700
14. Vilcek, J. and T.H. Lee (1991) *J. Biol. Chem.* 266:7313.
15. Warzocha, K., (1998). *Br. J. Cancer* 77,2357-2362
16. Yune TY., *et al.*, *J Neurotrauma.* (2004)21:1778-94.