DIACLONE
A Tepnel Company

Human TGF-β1 ELISA

650 010 096 **1 x 96 tests**
650 010 192 **2 x 96 tests**

INTENDED USE

The TGF-β1 ELISA is an enzyme-linked immunosorbent assay for the quantitative detection of human transforming growth factor beta-1 levels in cell culture supernatants, human serum, plasma, or other body fluids. **The TGF-β1 ELISA is for research use only. Not for use in diagnostic or therapeutic procedures.**

SUMMARY

Transforming growth factor-β (TGF-β) belongs to a family of dimeric 25 kDa polypeptides that are ubiquitously distributed in tissues and synthesized by many different cells (13). Three isoforms of transforming Growth Factor-β (TGF-beta1, beta-2 and beta-3) exist in mammals. They play critical roles in growth regulation and development. Each isoform is encoded by a unique gene on different chromosomes. All three of these growth factors are secreted by most cell types, generally in a latent form, requiring activation before they can exert biological activity. The TGF-betas possess three major activities: they inhibit proliferation of most cells, but can stimulate the growth of some mesechymal cells; they exert immunosuppressive effects and they enhance the formation of extracellular matrix. Two types of membrane receptors possessing kinase activity are involved in signal transduction. The TGF-betas are involved in wound repair processes and in starting inflammatory reaction and then in the resolution through chemotactic attraction of inflammatory cells and fibroblast (15).

TGF-β1 is the first recognized transforming growth factor (5), its subunits of each 12.5 kDa are bound via disulphide bridges. TGF-β1 is inhibitive to T- and B cell proliferation as well as to maturation and activation of macrophages. It furthermore inhibits activity of natural killer cells and lymphokine activated killer cells and blocks production of cytokines.

Measurement of TGF-β1 in blood has been advocated for diagnosis of various diseases. TGF-β1 has been shown to be an organizer of responses to neurodegeneration (10).

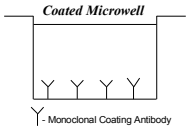
In this context, it turned out to be interesting in monitoring Alzheimer's disease (18), Down's syndrome, AIDS and Parkinson's disease (11). Serum and cerebrospinal fluid levels of Multiple Sclerosis patients were shown to be of great value to monitor remission and acute phases (4, 21). TGF-β1 is thought to play an important role in bone metabolism (22), it is considered a putative regulator of osteoclastic-osteoblastic interaction, thus it can be regarded as a marker for osteoporosis (14). TGF-β1 is involved in the pathogenesis of glomerular diseases (3, 23) such as diabetic nephropathy and glomerulosclerosis (28). TGF-β1 has been described to be functionally connected to major immune system abnormalities as in autoimmunity (SLE) (8). Serum levels have been shown to correlate with disease activity in autoimmune hepatitis (2). Elevated serum levels of TGF-β1 are determined in Chronic fatigue syndrome patients (6) and in Guillain-Baire syndrome patients (24). An inverse correlation with disease activity was described for TGF-β1 levels in Kawasaki disease (17) and patients with IgA deficiency (19).

TGF-β1 has been confirmed to promote fibrotic processes, thus it is implicated in the myelofibrosis with myeloid metaplasia (16). Increased serum levels of TGF-β1 in patients affected by thrombotic thromocytopenic purpura implicate its function on bone marrow haematopoiesis (29, 25). Determination of circulating TGF-β1 turned out to reflect the various stages in solid tumors as has been shown for cervical cancer (7), elevations were furthermore found in prostatic cancer (27), bladder cancer (9), and liver cancer (20).

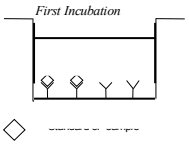
Decreased levels of TGF-β1 in the serum of sepsis and acute stroke patients (1, 12) may reflect the changing immunological-inflammatory status of these patients. Decreased TGF-β1 serum levels were described for patients with acute Plasmodium falciparum malaria (26).

PRINCIPLES OF THE TEST

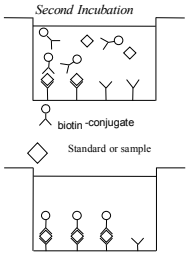
An anti-human TGF-β1 coating antibody is adsorbed onto microwells.



Human TGF-β1 present in the sample or standard binds to antibodies adsorbed to the microwells.



.A biotin-conjugated anti-human TGF-β1 antibody is added and binds to human TGF-β1 captured by the first antibody.



Following incubation unbound biotin-conjugated anti-human TGF-β1 antibody is removed during a wash step. Streptavidin-HRP is added and binds to the biotin-conjugated anti-human TGF-β1 antibody.
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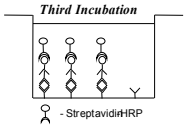
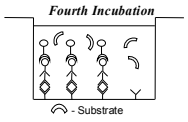
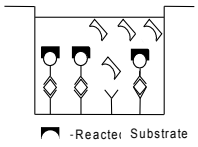


Following incubation unbound Streptavidin-HRP is removed during a wash step, and substrate solution reactive with HRP is added to the wells.



A coloured product is formed in proportion to the amount of human TGF-β1 present in the sample or standard. The reaction is terminated by addition of acid and absorbance is measured at 450 nm.



REAGENTS PROVIDED

REAGENTS (store at 2-8°C)	QTY 1 plate	QTY 1 plates	RECONSTITUTION
96-wells precoated microtiter plate	1	2	Ready-to-use
Plate covers	2	4	
Biotin-Conjugate anti-human TGF-β1 polyclonal antibody	1vial	2 vials	Dilute 100 times in Assay Buffer
Streptavidin-HRP	1vial	2vials	Dilute 100 times in Assay Buffer
TGF-β1 Standard	2 vials	4 vials	See label on the vial
Assay Buffer Concentrate*	1 vial	2 vials	(5 ml) 20X concentrate. Dilute in distilled water
Wash Buffer Concentrate*	1 bottle	2 bottles	(50 ml) 20X concentrate. Dilute in distilled water
Substrate Solution	1 vial	2 vials	(15 ml) Ready-to-use
Stop Solution (1 M Phosphoric acid)	1 vial	2 vials	(12 ml) Ready-to-use
1N HCl (pretreatment of samples)	1 vial	2 vials	(3 ml) Ready to use
1N NaOH (pretreatment of samples)	1 vial	2 vials	(3 ml) Ready to use
Blue Dye	1 vial	2 vials	(0.4 ml) Make a 1/250 dilution in the appropriate diluent
Green Dye	1 vial	2 vials	(0.4 ml) Make a 1/100 dilution in the appropriate diluent
Red dye	1 vial	2 vials	(0.4 ml) Make a 1/250 dilution in the appropriate diluent

* contains preservative

MATERIALS REQUIRED BUT NOT PROVIDED

- 5 ml and 10 ml graduated pipettes
- 5 µl to 1,000 µl adjustable single channel micropipettes with disposable tips
- 50 µl to 300 µl adjustable multichannel micropipette with disposable tips
- Multichannel micropipette reservoir
- Beakers, flasks, cylinders necessary for preparation of reagents
- Device for delivery of wash solution (multichannel wash bottle or automatic wash system)
- Microwell strip reader capable of reading at 450 nm (620 nm as optional reference wave length)
- Glass-distilled or deionized water
- Statistical calculator with program to perform linear regression analysis

SAFETY

- Reagents are intended for research use only and are not for use in diagnostic or therapeutic procedures.
- Do not mix or substitute reagents with those from other lots or other sources.
- Do not use kit reagents beyond expiration date on label.
- Do not expose kit reagents to strong light during storage or incubation.
- Do not pipette by mouth.
- Do not eat or smoke in areas where kit reagents or samples are handled.
- Avoid contact of skin or mucous membranes with kit reagents or specimens.
- Rubber or disposable latex gloves should be worn while handling kit reagents or specimens.
- Reagents containing thimerosal as preservative may be toxic if ingested.
- Avoid contact of substrate solutions with oxidizing agents and metal.
- Avoid splashing or generation of aerosols.
- In order to avoid microbial contamination or cross-contamination of reagents or specimens which may invalidate the test use disposable pipette tips and/or pipettes.
- Use clean, dedicated reagent trays for dispensing the conjugate and substrate reagents.
- Exposure to acids will inactivate the conjugate.
- Glass-distilled water or deionized water must be used for reagent preparation.
- Substrate solutions must be at room temperature prior to use.
- Decontaminate and dispose specimens and all potentially contaminated materials as if they could contain infectious agents.The preferred method of decontamination is autoclaving for a minimum of 1 hour at 121.5°C.
- Liquid wastes not containing acid and neutralized waste may be mixed with sodium hypochlorite in volumes such that the final mixture contains 1.0 % sodium hypochlorite. Allow 30 minutes for effective decontamination. Liquid waste containing acid must be neutralized prior to the addition of sodium hypochlorite.

STORAGE INSTRUCTIONS

Store kit reagents between 2° and 8°C. Immediately after use remaining reagents should be returned to cold storage (2° to 8°C). Expiry of the kit and reagents is stated on box front labels.
The expiry of the kit components can only be guaranteed if the components are stored properly, and if, in case of repeated use of one component, the reagent is not contaminated by the first handling.

SPECIMEN COLLECTION, PROCESSING AND STORAGE

Cell culture supernatants, human serum, plasma or other biological samples will be suitable for use in the assay. Remove serum from the clot or red cells, respectively, as soon as possible after clotting and separation.

Pay attention to a possible "**hook effect**" due to high sample concentrations (see chapter 11).

Samples containing a visible precipitate must be clarified prior to use in the assay. Do not use grossly hemolyzed or lipemic specimens.

Clinical samples should be kept at 2° to 8°C and separated rapidly before storing at -20°C to avoid loss of bioactivity. Avoid repeated freeze-thaw cycles.

PREPARATION OF REAGENTS

1. Wash Buffer

If crystals have formed in the Wash Buffer Concentrate, warm it gently until they have completely dissolved.

Pour entire contents (50 ml) of the **Wash Buffer Concentrate** into a clean 1000 ml graduated cylinder. Bring final volume to 1,000 ml with glass-distilled or deionized water. Mix gently to avoid foaming. The pH of the final solution should adjust to 7.4.

Transfer to a clean wash bottle and store at 2° to 25°C. Please note that the Wash Buffer is stable for 30 days. Wash Buffer may be prepared as needed according to the following table.

Number of Strips	Wash Buffer Concentrate (ml)	Distilled Water (ml)
1 - 6	25	475
1 - 12	50	950

2. Assay Buffer

Mix the contents of the bottle well. Add contents of **Assay Buffer Concentrate** (5.0 ml) to 95 ml distilled or deionized water and mix gently to avoid foaming. Store at 2° to 8°C. Please note that the Assay Buffer is stable for 30 days. Assay Buffer may be prepared as needed according to the following table:

Number of Strips	Assay Buffer Concentrate (ml)	Distilled Water (ml)
1 - 6	2.5	47.5
1 - 12	5.0	95.0

3. Prepararion of Biotin-Conjugate:

Please note that the biotin-conjugate should be used within 30 minutes after dilution.
Make a 1:100 dilution of the concentrated Biotin-Conjugate solution with Assay Buffer in a clean plastic tube as needed according to the following table:

Number of Strips	Biotin-Conjugate (µl)	Assay Buffer (ml)
1 - 6	30	2.97
1 - 12	60	5.94

4. Preparation of Streptavidin-HRP

Please note that the Streptavidin-HRP should be used within 30 minutes after dilution.
Make a 1:100 dilution of the concentrated **Streptavidin-HRP** solution with Assay Buffer in a clean plastic tube as needed according to the following table:

Number of Strips	Streptavidin-HRP (µl)	Assay Buffer (ml)
1 - 6	30	2.97
1 - 12	60	5.94

5. Preparation of Standard

Dilute TGF-β1 **Standard** by addition of distilled water. Dilution volume is stated on the label of the standard vial. Mix gently to ensure complete and homogeneous solubilization (concentration of reconstituted standard is 4000 pg/ml).Allow the standard to reconstitute for 10-30 minutes. Mix well prior to making dilution.
After use, remaining standard cannot be stored and has to be discarded.

5. Addition of Colour-giving Dyes

In order to help our customers to avoid any mistakes in pipetting, DIACLONE now offers a new tool that helps to monitor the addition of even very small volumes of a solution to the reaction well by giving distinctive colours to each step of the ELISA procedure.
This procedure is optional, does not in any way interfere with the test results, and is designed to help the customer with the performance of the test, but can also be omitted, just following the instruction booklet.
Alternatively, the dye solutions from the stocks provided (***Blue-Dye, Green-Dye, Red dye***) can be added to the reagents according to the following guidelines:

A. Diluent:

Before sample dilution add the ***Blue-Dye*** at a dilution of 1:250 (see table below) to the appropriate diluent (1x) according to the test protocol. After addition of ***Blue-Dye***, proceed according to the instruction booklet.

5 ml Assay Buffer	20 µl ***Blue-Dye***
12 ml Assay Buffer	48 µl ***Blue-Dye***
50 ml Assay Buffer	200 µl ***Blue-Dye***

B. Biotin-ConjugateHRP:

Before dilution of the concentrated Biotin-Conjugate, add the ***Green-Dye*** at a dilution of 1:100 (see table below) to the Assay Buffer (1x) used for the final conjugate dilution. Proceed after addition of ***Green-Dye*** according to the instruction booklet, preparation of Biotin-Conjugate.

3 ml Assay Buffer	30 µl ***Green-Dye***
6 ml Assay Buffer	60 µl ***Green-Dye***
12 ml Assay Buffer	120 µl ***Green-Dye***

C. Streptavidin-HRP:

Before dilution of the concentrated Streptavidin-HRP, add the ***Red-Dye*** at a dilution of 1:250 (see table below) to the Assay Buffer (1x) used for the final Streptavidin-HRP dilution. Proceed after addition of ***Red-Dye*** according to the instruction booklet, preparation of Streptavidin-HRP.

6 ml Assay Buffer	24 µl ***Red-Dye***
12 ml Assay Buffer	48 µl ***Red-Dye***

TEST PROTOCOL

a. Prepare your samples before starting with the test procedure.
Dilute serum, plasma and cell culture samples 1:10 with Assay Buffer (180 µl Assay Buffer + 20 µl sample).
Add 20 µl 1N HCl to 200 µl of prediluted sample, mix and incubate for 1 hour at room temperature.
Neutralize by addition of 20 µl 1N NaOH.

b. Mix all reagents thoroughly without foaming before use.

c. Determine the number of microwell strips required to test the desired number of samples plus appropriate number of wells needed for running blanks and standards. Each sample, standard, blank, and optional control sample should be assayed in duplicate. Remove sufficient **Microwell Strips coated with Antibody** to human TGF-β1 from the aluminium pouch immediately prior to use. Return any wells not required for this assay with desiccant to the pouch. Seal tightly.

d. Wash the microwell strips three times with approximately 300 µl Wash Buffer per well with thorough aspiration of microwell contents between washes. Take care not to scratch the surface of the microwells. After the last wash, tap microwell strips on absorbent pad or paper towel to remove excess Wash Buffer. Use the microwell strips immediately after washing or place upside down on a wet absorbent paper for not longer than 15 minutes. Do not allow wells to dry.

e. Add 100 µl of Assay Buffer in duplicate to all standard wells. Prepare standard dilutions by pipetting 100µl of **TGF-β1 Standard**, in duplicate, into well A1 and A2 (see Figure 1 and 2). Mix and transfer 100µl to wells B1 and B2 respectively. Take care not to scratch the inner surface of the microwells. Mix the contents of well B1 and B2 and transfer 100 µl to well C1 and C2 respectively. Continue this procedure four times, creating two rows of TGF-β1 Standard dilutions ranging from 30-0.5 ng/ml. Discard 100 µl of the contents from the last microwell used (G1, G2).

Figure 1. Preparation of TGF-β1 standard dilutions:

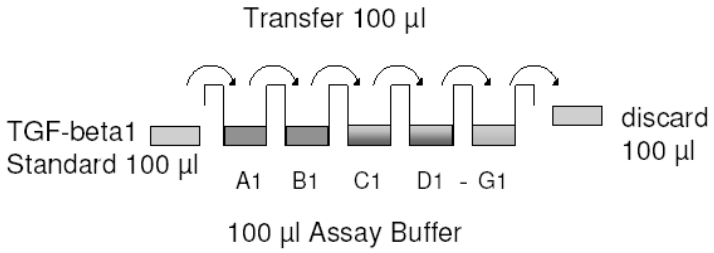


Figure 2. Diagram depicting an example of the arrangement of blanks, standards and samples in the microwell strips:

	Standard Concentrations ng/mL		Sample wells									
	1	2	3	4	5	6	7	8	9	10	11	12
A	2000	2000										
B	1000	1000										
C	500	500										
D	250	250										
E	125	125										
F	62.5	62.5										
G	31.25	31.25										
H	Blank	Blank										

f. Add 100 µl of **Assay Buffer,** in duplicate, to the blank wells.

g. Add 60 µl of **Assay Buffer** to the **sample wells**

h. Add 40 µl of each pretreated **Sample**, in duplicate, to the designated wells

i. Cover with a **Plate Cover** and incubate at room temperature (18ºC to 25ºC) for **2 hours** on **a rotator set at 100 rpm.**

j. Prepare **Biotin-Conjugate.**

k. Remove **Plate Cover** and empty wells. Wash microwell strips 5 times according to point d. of the test protocol. Proceed immediately to the next step.

l. Add 100 µl of **Biotin-Conjugate** to all wells, including the blank wells.

m. Cover with a **Plate Cover** and incubate at room temperature (18ºC to 25ºC) for **1 hour** on **a rotator set at 100 rpm.**

n. Prepare **Streptavidin-HRP**

o. Remove **Plate Cover** and empty wells. Wash microwell strips 5 times according to point d. of the test protocol. Proceed immediately to the next step.

p. Add 100 µl of diluted **Streptavidin-HRP** to all wells, including the blank wells.

q. Cover with a **Plate Cover** and incubate at room temperature (18ºC to 25ºC) for **1 hour** on **a rotator set at 100 rpm.**

r. Remove **Plate Cover** and empty wells. Wash microwell strips 5 times according to point d. of the test protocol. Proceed immediately to the next step.

s. Pipette 100 µl of **TMB Substrate Solution** to all wells, including the blank wells.

t. Incubate the microwell strips at room temperature (18° to 25°C) for about 30 minutes, if available on a rotator set at 100 rpm. Avoid direct exposure to intense light.
The point at which the substrate reaction is stopped is often determined by the ELISA reader being used. Many ELISA readers record absorbance only up to 2.0 O.D. **The O.D. values at the plate should be monitored and the substrate reaction stopped before positive wells are no longer properly recordable.**

u. Stop the enzyme reaction by pipetting 100 µl of **Stop Solution** into each well, including the blank wells. It is important that the Stop Solution is spread quickly and uniformly throughout the microwells to completely inactivate the enzyme. Results must be read immediately after the Stop Solution is added or within one hour if the microwell strips are stored at 2 - 8°C in the dark.

v. Read absorbance of each microwell on a spectro-photometer using 450 nm as the primary wave length (optionally 620 nm as the reference wave length; 610 nm to 650 nm is acceptable). Blank the plate reader according to the manufacturer's instructions by using the blank wells. Determine the absorbance of both, the samples and the TGF-β1 standards.

CALCULATION OF RESULTS

- Calculate the average absorbance values for each set of duplicate standards and samples. Duplicates should be within 20 per cent of the mean.

- Create a standard curve by plotting the mean absorbance for each standard concentration on the ordinate against the TGF-β1 concentration on the abscissa. Draw a best fit curve through the points of the graph.

- To determine the concentration of circulating TGF-β1 for each sample, first find the mean absorbance value on the ordinate and extend a horizontal line to the standard curve. At the point of intersection, extend a vertical line to the abscissa and read the corresponding TGF-β1 concentration.

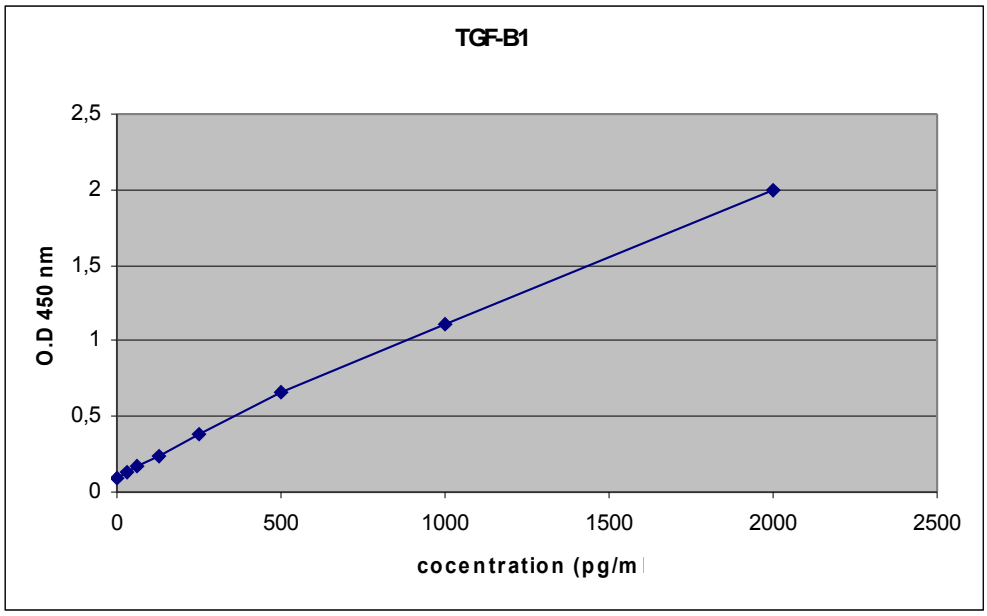
For samples which have been diluted according to the insructions given in this manual (e.g. 1:12) the concentration read from the standard curve must be multiplied by the dilution factor (e.g. x12).

Note: Calculation of samples with an O.D. exceeding 2.0 may result in incorrect, low TGF-β1 levels. Such samples require further dilution with Standard / Sample Diluent in order to precisely quantitate the actual TGF-β1 level.

It is suggested that each testing facility establishes a control sample of known TGF-β1 concentration and runs this additional control with each assay. If the values obtained are not within the expected range of this control, the assay results may be invalid.

- A representative standard curve is shown in Figure 3. This curve cannot be used to derive test results. Every laboratory must prepare a standard curve for each group of microwell strips assayed.

Figure 3. Representative standard curve for TGF-β1 ELISA. Recombinant soluble TGF-β1 was diluted in Assay Buffer; symbols represent the mean of three parallel titrations. Do not use this standard curve to derive test results. A standard curve must be run for each group of microwell strips assayed.



Typical data using the TGF-β1ELISA

Measuring wavelength: 450 nm
Reference wavelength: 620 nm

Standard	TGF-β1 Concentration (ng/ml)	O.D. Mean	C.V (%)
1	2000 2000	2000	0.2
2	1000 1000	1105	4.1
3	500 500	0.665	0.4
4	250 250	0.390	0.6
5	125 125	0.244	0.2
6	62.5 62.5	0.168	1.6
7	31.25	0.131	0.2
	31.25		
Blank	0 0	0.098	0.3

LIMITATIONS OF THE PROCEDURE

- Since exact conditions may vary from assay to assay, a standard curve must be established for every run.
- Bacterial or fungal contamination of either samples or reagents or cross-contamination between reagents may cause erroneous results.
- Disposable pipette tips, flasks or glassware are preferred, reusable glassware must be washed and thoroughly rinsed of all detergents before use.
- Improper or insufficient washing at any stage of the procedure will result in either false positive or false negative results. Completely empty wells before dispensing fresh Wash Buffer, fill with Wash Buffer as indicated for each wash cycle and do not allow wells to sit uncovered or dry for extended periods.

PERFORMANCE CHARACTERISTICS

Sensitivity

The limit of detection for recombinant TGF-β1, spiked into normal human serum, defined as the analyte concentration resulting in an absorption significantly higher than the absorption of the dilution medium (mean plus two standard deviations) was determined to be 9 pg/ml (mean of three independent assays).

Reproducibility

a. Intra-assay

Intra-assay variability was determined by 6 replicates of 8 serum samples. The average coefficient of variation was 5.1%.

Positive Sample	Experiment	VEGF-A Concentration (pg/ml)	Coefficient of Variation (%)
1	1	45241	3.3
	2	41522	4.5
	3	42803	3.5
2	1	24734	10.6
	2	22431	1.8
	3	22822	2.0
3	1	10409	6.2
	2	11284	1.9
	3	13140	5.6
4	1	6903	8.2
	2	5919	8.0
	3	5673	4.0
5	1	8313	7.2
	2	9488	2.5
	3	9559	2.6
6	1	12299	6.8
	2	10982	4.7
	3	13200	2.5
7	1	5861	10.6
	2	5427	4.5
	3	5984	2.0
8	1	2953	8.0
	2	3543	6.9
	3	3808	5.6

b. Inter-assay

Inter-assay variability was determined by 18 replicates of 8 serum samples. The average coefficient of variation was 8.4 %.

Sample	TGF-□1 Concentration (pg/ml)	Coefficient of Variation (%)
1	43189	4.4
2	23329	5.3
3	11611	12.0
4	6165	10.6
5	9120	7.7
6	12160	9.2
7	5757	5.1
8	3435	12.7

Specificity

The assay detects both natural and recombinant human TGF-β1 . No cross reactivity was apparent with human serum factors spiked into human serum, notably there was no cross reactivity with human TGF-β2 and TGF-β3.

Spike Recovery

The spike recovery was evaluated by spiking 2 levels of human human TGF-β1 TGF-β1into serum, plasma and cells culture supernatant. Recoveries were determined with 4 replicates each. The amount of endogenous human
TGF-β1 in unspiked samples was subtracted from the spike values.

Sample matrix	Spike high (%)	Spike medium (%)
Serum	84	83
Plasma (EDTA)	74	110
Plasma (citrate)	91	97
Plasma (heparin)	97	90
Cell culture supernatant	124	141

Dilution Linearity

Serum, plaama and cell culture supernatant samples with different levels of human TGF-β1were analysed at serial 2 fold dilutions with 4 replicates each.

Sample matrix	.Recovery of Exp. Val	
	Range (%)	Mean (%)
Serum	88-107	97
Plasma (EDTA)	108-150	125
Plasma (citrate)	109-151	132
Plasma (heparin)	52-81	67
Cell culture supernatant	90-115	106

Sample stability

a. Freeze-Thaw Stability

Aliquots of serum samples (unspiked or spiked) were stored at -20°C and thawed up to 5 times, and the human TGF-β1 levels determined. There was no significant loss of human TGF-β1 immunoreactivity detected by freezing or thawing.

b. Storage stability

Aliquots of a serum sample (spiked or unspiked) were stored at -20°C, 2-8°C, room temperature (RT) and at 37°C, and the human TGF-β1 level determined after 24 h. There was no significant loss of human TGF-β1 immunoreactivity detected during storage inder above conditions.

Expected values

A panel of 40 serum samples from randomly selected apparently healthy donors (males and females) was tested for human TGF-β1.

Sample matrix	Number of samples evaluated	Range (pg/ml)	Mean (pg/ml)	Standard deviation (pg/ml)
Serum	40	4639-14757	8225	2218
Plasma (EDTA)	40	2404-25558	6479	3778
Plalma (citrate)	40	6518-31737	16942	5762
Plasma (Heparin)	40	3081-15879	6580	2882

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REAGENT PREPARATION SUMMARY

A. Wash Buffer Add **Wash Buffer Concentrate** 20 x (50 ml) to 950 ml distilled water

B. Assay Buffer

Number of Strips	Assay Buffer Concentr. (ml)	Distilled Water (ml)
1 - 6	2.5	47.5
1 - 12	5.0	95.0

C. Standard Reconstitute **Standard** by addition of distilled water. Reconstitution volume is stated on label of the standard vial.

D. Biotin-Conjugate Make a 1:100 dilution according to the table.

Number of Strips	Biotin-Conjugate (µl)	Assay Buffer (ml)
1 - 6	30	2.97
1 - 12	60	5.94

E. Streptavidin-HRP

Number of Strips	Streptavidin-HRP (µl)	Assay Buffer (ml)
1 - 6	30	2.97
1 - 12	60	5.94

TEST PROTOCOL SUMMARY

- Pretreat Samples
- Wash Microwell Strips twice with Wash Buffer
- Add 100 µl Assay Buffer, in duplicate, to all standard wells
- Pipette 100 µl TGF-β1 Standard into the first standard wells and create standard dilutions ranging from 30 to 0.5 ng/ml by transferring 100 µl from well to well; discard 100µl from the last wells.
- Add 100 µl Assay Buffer, in duplicate, to the blank wells
- Add 60 µl Assay Buffer to sample wells.
- Add 40 µl sample in duplicate to designated sample wells.
- Cover microwell strips and incubate **2 hours** at room temperature (18° to 25°C) on a rotator set at 100 rpm
- Prepare Biotin-Conjugate Conjugate Mixture.
- Empty and wash microwell strips 5 times with Wash Buffer
- Add 100 µl of Biotin-Conjugate to all wells.
- Cover microwell strips and incubate **1 hour** at room temperature (18° to 25°C) on a rotator set at 100 rpm
- Empty and wash microwell strips 5 times with Wash Buffer
- Add 100 µl of diluted HRP-Conjugate to all wells
- Cover microwell strips and incubate **1 hour** at room temperature (18° to 25°C) on a rotator set at 100 rpm
- Empty and wash microwell strips 5 times with Wash Buffer
- Add 100 µl of TMB Substrate Solution to all wells including blank wells
- Incubate the microwell strips for about **30 minutes** at room temperature (18° to 25°C)
- Add 100 µl Stop Solution to all wells including blank wells
- Blank microwell reader and measure colour intensity at 450 nm

- **Note:If instructions in this protocol have been followed samples have been diluted 1:30 (20µl sample + 180µl Assay Buffer + 20µl 1N HCL + 20µl 1 N NaOH and 40 µl pretreated sample + 60µl Assay Buffer), the concentration read from the standard curve must be multiplied by the dilution factor (x30).**