Human TGFβ1 ELISA Kit

Instructions for use

Catalogue numbers: 1x96 tests: 650.010.096

For research use only

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Human TGFβ1 ELISA KIT

1. Intended use

The Diaclone TGFβ1 ELISA kit is a solid phase sandwich ELISA for the *in-vitro* qualitative and quantitative determination of TGFβ1 in supernatants, buffered solutions or serum and plasma samples. This assay will recognise both natural and recombinant human TGFβ1.

This kit has been configured for research use only. Not suitable for use in therapeutic procedures.

2. Introduction

2.1. 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).

2.2. Principle of the method

A capture Antibody highly specific for TGFβ1 has been coated to the wells of the microtiter strip plate provided during manufacture. Binding of TGFβ1 in samples and known standards to the capture antibodies is completed and then any excess unbound analyte is removed.
During the next incubation period the binding of the Biotin-conjugated anti- TGFβ1 antibody to the analyte occurs. Any excess unbound secondary antibody is then removed.
The HRP conjugate solution is then added to every well including the zero wells, following incubation excess conjugate is removed by careful washing.
A chromogen substrate is added to the wells resulting in the progressive development of a blue coloured complex with the conjugate. The colour development is then stopped by the addition of acid turning the resultant final product yellow. The intensity of the produced coloured complex is directly proportional to the concentration of TGFβ1 present in the samples and standards.
The absorbance of the colour complex is then measured and the generated OD values for each standard are plotted against expected concentration forming a standard curve. This standard curve can then be used to accurately determine the concentration of TGFβ1 in any sample tested.

3. Reagents provided and reconstitution

Reagents (Store@2-8°C)	**Quantity 1x96 well kit** Cat no. 650.010.096	**Reconstitution**
96 well precoated microtiter plate	1	Ready to use
plate covers	2	n/a
TGFβ1 Standard	2	Reconstitute as directed on the vial (see Assay preparation, section 8)
Biotin-conjugate anti- TGFβ1	1 (120µl)	Dilute 100 times in Assay Buffer (see Assay preparation, section 8)
Streptavidin-HRP	1 (150µl)	Dilute 100 times in Assay Buffer (see Assay preparation, section 8)
Assay Buffer	1 (5ml)	20X concentrate dilute in distilled water (see Assay preparation, section 8)
Wash Buffer	1 (50ml)	20X concentrate dilute in distilled water (see Assay preparation, section 8)
Substrate Solution	1 (15ml)	Ready to use
Stop solution	1 (15ml)	Ready to use

4. Materials required but not provided

- 1N NaOH and 1 N HCL are needed to run the test
- Microtiter plate reader fitted with appropriate filters (450 nm required with optional 620 nm reference filter)
- Microplate washer or wash bottle
- 10, 50, 100, 200 and 1,000µl adjustable single channel micropipettes with disposable tips
- 50-300µl multi-channel micropipette with disposable tips
- Multichannel micropipette reagent reservoirs
- Distilled water
- Vortex mixer
- Miscellaneous laboratory plastic and/or glass, if possible sterile

5. Storage Instructions

Store kit reagents between 2 and 8°C. Immediately after use remaining reagents should be returned to cold storage (2-8°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.

Wash Buffer 1X: Once prepared, store at 2-8°C for up to 1 month.

Assay Buffer 1X: Once prepared, store at 2-8°C for up to 1 month.

Reconstituted Standard: Once prepared use immediately and do not store.

Diluted Biotin conjugate Anti-TGFβ1: Once prepared use immediately and do not store.

Diluted Streptavidin-HRP: Once prepared use immediately and do not store.

6. Specimen collection, processing & 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.

Cell culture supernatants: Remove particulates and aggregates by spinning at approximately 1000 x g for 10 min.

Serum: Use pyrogen/endotoxin free collecting tubes. Serum should be removed rapidly and carefully from the red cells after clotting. Following clotting, centrifuge at approximately 1000 x g for 10 min and remove serum.

Plasma: EDTA, citrate and heparin plasma can be assayed. Spin samples at 1000 x g for 30 min to remove particulates. Harvest plasma.

Storage: If not analyzed shortly after collection, samples should be aliquoted (250-500µl) to avoid repeated freeze-thaw cycles and stored frozen at –70°C. Avoid multiple freeze-thaw cycles of frozen specimens.

Recommendation: Do not thaw by heating at 37°C or 56°C. Thaw at room temperature and make sure that sample is completely thawed and homogeneous before use. When possible avoid use of badly haemolysed or lipemic sera. If large amounts of particles are present these should be removed prior to use by centrifugation or filtration.

Note: Pay attention to a possibly elevated blank signal in cell culture supernatant samples containing serum components (e.g. FCS), due to latent TGF beta levels in animal serum.

7. Safety & precautions for use

- Handling of reagents, serum or plasma specimens should be in accordance with local safety procedures, e.g. CDC/NIH Health manual : "Biosafety in Microbiological and Biomedical Laboratories" 1984.
- Laboratory gloves should be worn at all times.
- Avoid any skin contact with Stop solution and substrate solution. In case of contact, wash thoroughly with water.
- Do not eat, drink, smoke or apply cosmetics where kit reagents are used.
- Do not pipette by mouth.
- When not in use, kit components should be stored refrigerated as indicated on vials or bottles labels.
- All reagents should be warmed to room temperature before use. Lyophilized standards should be discarded after use.
- Once the desired number of strips has been removed, immediately reseal the bag to protect the remaining strips from deterioration.
- Cover or cap all reagents when not in use.
- Do not mix or interchange reagents between different lots.
- Do not use reagents beyond the expiration date of the kit.
- Use a clean disposable plastic pipette tip for each reagent, standard, or specimen addition in order to avoid cross contamination, for the dispensing of Stop solution and Substrate Solution, avoid pipettes with metal parts.
- Use a clean plastic container to prepare the washing solution.
- Thoroughly mix the reagents and samples before use by agitation or swirling.
- All residual washing liquid must be drained from the wells by efficient aspiration or by decantation followed by tapping the plate forcefully on absorbent paper. Never insert absorbent paper directly into the wells.
- The Substrate Solution is light sensitive. Avoid prolonged exposure to light. Also, avoid contact of the TMB solution with metal to prevent colour development. Warning substrate Solution is toxic avoid direct contact with hands. Dispose off properly.
- If a dark blue colour develops within a few minutes after preparation, this indicates that the substrate solution has been contaminated and must be discarded. Read absorbance’s within 1 hour after completion of the assay.
- When pipetting reagents, maintain a consistent order of addition from well-to-well. This will ensure equal incubation times for all wells.
- Follow incubation times described in the assay procedure.
- Dispense the Substrate Solution within 15 min of the washing of the microtiter plate.

8. Assay Preparation

Bring all reagents to room temperature before use

8.1. Assay Design

Determine the number of microwell strips required to test the desired number of samples plus appropriate number of wells needed for running zeros and standards. Each sample, standard and zero should be tested **in duplicate**. Remove sufficient microwell strips for testing from the pouch immediately prior to use. Return any wells not required for this assay with desiccant to the pouch. Seal tightly and return to 2-8ºC storage.

Example plate layout (example shown for a 6 point standard curve)

	Standards		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	Blank	Blank										
H												

All remaining empty wells can be used to test samples in duplicate

8.2. Preparation of 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 1,000 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.

8.3. Preparation of 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.

8.4. Preparation of Standard

Standard vials must be reconstituted with the volume of distilled water shown on the vial immediately prior to use. This reconstitution gives a stock solution of 4000pg/ml of TGFβ1. **Mix the reconstituted standard gently by inversion only**. Serial dilutions of the standard are made directly in the assay plate to provide the concentration range from 2000 to 62.5 pg/ml. A fresh standard curve should be produced for each new assay.

- Add 100µl of Assay buffer to all standard and blank wells
- Immediately after reconstitution add 100µl of the reconstituted standard to wells A1 and A2, which provides the highest concentration standard at 2000pg/ml. Mix the well contents by repeated aspirations and ejections taking care not to scratch the inner surface of the wells
- Transfer 100µl from wells A1 and A2 to B1 and B2. Mix the well contents by repeated aspirations and ejections taking care not to scratch the inner surface of the wells
- Continue this 1:1 dilution using 100µl from wells B1 and B2 through to wells F1 and F2 providing a serial diluted standard curve ranging from 2000 to 62.5pg/ml
- Discard 100µl from the final wells of the standard curve (F1 and F2)

Alternatively these dilutions can be performed in separate clean tubes and immediately transferred directly into the relevant wells.

8.5. Preparation of Samples

Prepare your samples before starting with the test procedure.

Dilute serum, plasma and cell culture samples 1:10 with Assay Buffer 1X (180 µl Assay Buffer + 20 µl sample).
Add 20 µl 1N HCl (see “Materials required but not provided”) to 200 µl of prediluted sample, mix and incubate for 1 hour at room temperature.
Neutralize by addition of 20 µl 1N NaOH (see “Materials required but not provided”).
Vortex!

8.6. Preparation of Biotin Conjugate

It is recommended this reagent is prepared immediately before use.

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	60	5.94
1 - 12	120	11.88

8.7. Preparation of Streptavidin-HRP

It is recommended this reagent is prepared immediately before use.

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	60	5.94
1 - 12	120	11.88

9. Method

We strongly recommend that every vial is mixed thoroughly without foaming prior to use.

Prepare all reagents as shown in section 8.
Note: final preparation of Biotin conjugate (section 8.6) and Streptavidin-HRP (section 8.7) should occur immediately before use.

Assay Step		Details
1.	Wash	Remove the pre-coated plate from the sealed pouch, removed any un-needed strips and wash the plate as follows: a) Dispense 0.3 ml of **1x washing solution** into each well b) Aspirate the contents of each well c) Repeat step a and b another two times
2.	Addition	**Prepare Standard curve** as shown in section 8.4
3.	Addition	Add **60µl** of **Assay Buffer** and **40µl** of each **pre-treated sample** (see section 8.5, it is absolutely necessary to vortex the samples!) in duplicate to appropriate number of wells
4.	Incubation	Cover with a plastic plate cover and incubate at room temperature (18 to 25°C) for **2 hours** on a microplate shaker set at 400rpm. (Shaking is absolutely necessary for an optimal test performance.)
5.	Wash	Remove the cover and wash the plate as follows: a) Aspirate the liquid from each well b) Dispense 0.3 ml of **1x washing solution** into each well c) Aspirate the contents of each well d) Repeat step b and c another two times
6.	Addition	Add 100µl of diluted **biotin Conjugate** to all wells
7.	Incubation	Cover with a plastic plate cover and incubate at room temperature (18 to 25°C) for **1 hour** on a microplate shaker set at 400rpm. (Shaking is absolutely necessary for an optimal test performance.)
8.	Wash	Repeat wash step 5.
9.	Addition	Add 100µl of diluted **Streptavidin-HRP** solution into all wells
10.	Incubation	Cover with a plastic plate cover and incubate at room temperature (18 to 25°C) for **1 hour** on a microplate shaker set at 400rpm. (Shaking is absolutely necessary for an optimal test performance.)
11.	Wash	Repeat wash step 5.
12.	Addition	Add 100µl of ready-to-use **Substrate Solution** into all wells
13.	Incubation	Incubate for **30 minutes*** at room temperature. Avoid direct exposure to light by wrapping the plate in aluminium foil
14.	Addition	Add 100µl of **Stop solution** into all wells
Read the absorbance value of each well (immediately after step 14.) on a spectrophotometer using 450 nm as the primary wavelength and optionally 620 nm as the reference wave length (610 nm to 650 nm is acceptable).		

**Incubation time of the substrate solution is usually determined by the ELISA reader performance. Many ELISA readers only record absorbance up to 2.0 O.D. Therefore the colour development within individual microwells must be observed by the analyst, and the substrate reaction stopped before positive wells are no longer within recordable range.*

10. Data Analysis

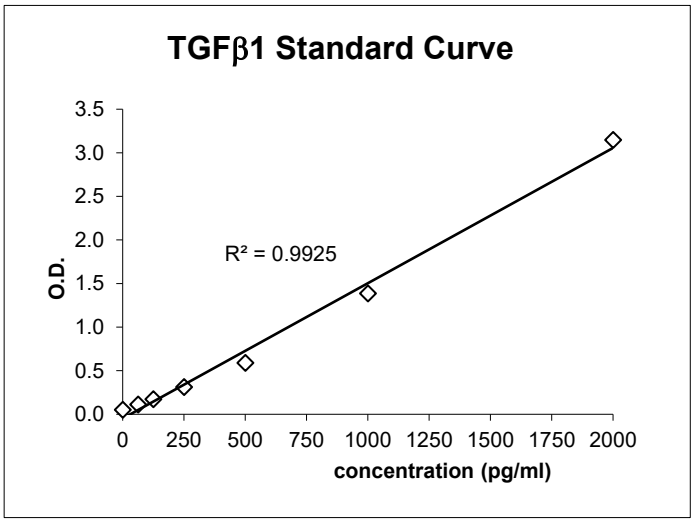
Calculate the average absorbance values for each set of duplicate standards, controls and samples. Ideally duplicates should be within 20% of the mean.

Generate a linear standard curve by plotting the average absorbance of each standard on the vertical axis versus the corresponding TGFβ1 standard concentration on the horizontal axis.

The amount of TGFβ1 in each sample is determined by extrapolating OD values against TGFβ1 standard concentrations using the standard curve**.**

Example TGFβ1 Standard Curve

Standard	TGFβ1 Conc (pg/ml)	OD (450nm) Mean	CV (%)
1	2000	3.148	2.5
2	1000	1.387	1.0
3	500	0.590	0.4
4	250	0.313	4.1
5	125	0.172	3.5
6	62.5	0.112	2.0
Zero	0	0.051	2.0



Note: curve shown above should not be used to determine results. Every laboratory must produce a standard curve for each set of microwell strips assayed.

For samples which have been diluted according to the protocol (1:30), the calculated concentration should be multiplied by the dilution factor.

11. Assay limitations

Do not extrapolate the standard curve beyond the maximum standard curve point. The dose-response is non-linear in this region and good accuracy is difficult to obtain. Concentrated samples above the maximum standard concentration must be diluted with Standard diluent or with your own sample buffer to produce an OD value within the range of the standard curve. Following analysis of such samples always multiply results by the appropriate dilution factor to produce actual final concentration.

The influence of various drugs on end results has not been investigated. Bacterial or fungal contamination and laboratory cross-contamination may also cause irregular results.

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 Washing Buffer, fill with Washing Buffer as indicated for each wash cycle and do not allow wells to sit uncovered or dry for extended periods.

Disposable pipette tips, flasks or glassware are preferred, reusable glassware must be washed and thoroughly rinsed of all detergents before use.

As with most biological assays conditions may vary from assay to assay therefore **a fresh standard curve must be prepared and run for every assay.**

12. Performance Characteristics

12.1. Sensitivity

The limit of detection for human TGFβ1 defined as the analyte concentration resulting in an absorbance significantly higher than that of the dilution medium (mean plus 2 standard deviations) was determined to be 8.6 pg/ml (mean of 6 independent assays).

12.2. Specificity

The assay detects both natural and recombinant human TGFβ1. The cross-reactivity of TGFβ2 and TGFβ3, and of TNFβ, IL-8, IL-6, IL-2, TNFα, IL-1β, IL-4, IFNγ, IL12p70, IL-5 and IL-10 was evaluated by spiking these proteins at physiologically relevant concentrations into serum. No cross-reactivity was detected.

12.3. Precision

Intra-assay
Reproducibility within the assay was evaluated in three independent experiments. Each assay was carried out with 6 replicates of 8 serum samples containing different concentrations of TGFβ1.
The calculated overall coefficient of variation was 3.2%.

Inter-assay
Assay to assay reproducibility within one laboratory was evaluated in three independent experiments. Each assay was carried out with 6 replicates of 8 serum samples containing different concentrations of TGFβ1:
The calculated overall coefficient of variation was 4.9%.

12.4. Dilution Parallelism

Serum, plasma and cell culture supernatant samples with different levels of human TGF-β1 were analysed at serial 2 fold dilutions with 4 replicates each.

Sample matrix	Recovery of Exp. Val.	
	Mean (%)	Range (%)
Serum	104	97-112
Plasma (EDTA)	129	119-138
Plasma (citrate)	119	108-130
Plasma (heparin)	126	121-130
Cell culture supernatant	106	95-118

12.5. Spike Recovery

The spike recovery was evaluated by spiking 3 levels of human TGFβ1 into serum, plasma and cells culture supernatant. Recoveries were determined with 4 replicates each. The amount of endogenous human TGFβ1 in unspiked samples was subtracted from the spike values.

Sample matrix	Spike high (%)	Spike medium (%)	Spike low (%)
Serum	85	97	96
Plasma (EDTA)	90	84	82
Plasma (citrate)	108	92	92
Plasma (heparin)	110	87	93
Cell culture supernatant	87	85	95

12.6. Stability

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 TGFβ1 level determined after 24 h. There was no significant loss of human TGFβ1 immunoreactivity detected during storage under above conditions.

Freeze-thaw Stability
Aliquots of serum samples (unspiked or spiked) were stored at -20°C and thawed 5 times, and the human TGFβ1 levels determined. There was no significant loss of human TGFβ1 immunoreactivity detected by freezing or thawing.

12.7. Expected serum values

A panel of samples from randomly selected apparently healthy donors (males and females) was tested for human TGFβ1.

Sample matrix	Number of samples evaluated	Range (pg/ml)	Mean (pg/ml)	Standard deviation (pg/ml)
Serum	16	5222-13731	6723	1978
Plasma (EDTA)	40	0-2644	729	389
Plasma (citrate)	40	908-3378	1726	578
Plasma (Heparin)	40	0-377	46	96

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14. Assay Summary

Total procedure length : 4h30min

Add pre-treated sample and diluted standard

↓

Incubate 2 hours at room temperature

↓

Wash three times

↓

Add 100µl of biotin conjugate

↓

Incubate 1 hour at room temperature

↓

Wash three times

↓

Add 100µl of Streptavidin-HRP

↓

Incubate 1 hour at room temperature

↓

Wash three times

↓

Add 100µl of Substrate Solution
Protect from light. Let the color develop for 30 min.

↓

Add 100µl Stop solution

↓

Read Absorbance at 450 nm

Supplier:

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