Rat TGF-β1 ELISA Kit

Instructions for use

Catalogue numbers: 1x96 tests: 670.020.096
2x96 tests: 670.020.192

For research use only

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Version 7 24/01/2013

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Rat TGF-β1 ELISA KIT

1. Intended use

The rat TGF-β1 ELISA is an Enzyme-Linked ImmunoSorbent Assay for the quantitative detection of rat transforming growth factor beta-1 levels in cell culture supernatants, rat serum, plasma, or other body fluids.

This kit has been configured for research use only.

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 (2). 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 TGFbetas 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 (3).

TGF-β1 is the first recognized transforming growth factor (1), 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.

2.2. Principle of the method

An anti-rat TGF-β1 (rTGF-β1) coating antibody is adsorbed onto microwells.

rTGF-β1 present in the sample or standard binds to antibodies adsorbed to the microwells; a biotin-conjugated monoclonal anti-rTGF-β1 antibody is added and binds to rTGF-β1 captured by the first antibody

Following incubation unbound biotin conjugated anti-rTGF-β1 is removed during a wash step. Streptavidin-HRP is added and binds to the biotin conjugated anti-rTGF-β1. 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 rTGF-β1 present in the sample. The reaction is terminated by addition of acid and absorbance is measured at 450nm. A standard curve is prepared from seven rTGF-β1 standard dilutions and rTGF-β1 sample concentration determined.

3. Reagents provided and reconstitution

REAGENTS (store at 2-8°C)	Quantity 1x96 well kit 670.020.096	Quantity 2x96 well kit 670.020.192	RECONSTITUTION
96-wells precoated microtiter plate	1	2	Ready-to-use
Plate covers	2	4	
Biotin-Conjugate anti-rat TGF-β1 polyclonal antibody	1 vial	2 vials	Dilute 100 times in Assay Buffer (120µl)
Streptavidin-HRP	1 vial	2 vials	Dilute 100 times in Assay Buffer (150µl)
Rat TGF-β1 Standard	2 vials	4 vials	See label on the vial
Assay Buffer Concentrate	2 vial	4 vials	(5 ml) 20X concentrate. Dilute in distilled water
Wash Buffer Concentrate	1 vial	2 vials	(50 ml) 20X concentrate. Dilute in distilled water
Substrate Solution	1 vial	2 vials	(15 ml) Ready-to-use
Stop Solution (1M 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

4. Materials required but not provided

- Microtiter plate reader fitted with appropriate filters (450nm required with optional 630nm 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. Specimen collection, processing & storage

Cell culture supernatants, 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.

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.

6. 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 H_2SO_4 and TMB. 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 or frozen 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 H_2SO_4 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 TMB solution is light sensitive. Avoid prolonged exposure to light. Also, avoid contact of the TMB solution with metal to prevent colour development. Warning TMB 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 TMB 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 TMB solution within 15 min of the washing of the microtitre plate

7. Assay Preparation

Bring all reagents to room temperature before use

7.1. 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 aluminium pouch immediately prior to use. Return any wells not required for this assay with desiccant to the pouch. Seal tightly and return to 2-8°C 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

7.2. 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. 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

7.3. 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. 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

7.4. 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 mTGF-β1. **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 62.5pg/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.

7.5. Preparation of Samples

Prepare your samples before starting with the test procedure. Dilute serum, plasma and cell culture samples 1:20 with Assay Buffer (190 µl Assay Buffer + 10 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.

7.6. Preparation 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	60	5.94
1 - 12	120	11.88

7.7. 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	60	5.94
1 - 12	120	11.88

7.8. Addition of Color 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***

8. Method

We strongly recommend that every vial is mixed thoroughly without foaming prior to use except the standard vial which must be mixed gently by inversion only.

Prepare all reagents and pretreat samples as shown in section 7.

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
2.	Addition	**Prepare Standard curve** as shown in section 7.4
3.	Addition	Add 100µl of Assay Buffer in duplicate to the blank wells Add 100µl of pre treated Sample in designated wells (see section 7.5)
4.	Incubation	Cover with a plastic plate cover and incubate at room temperature (18ºC to 25ºC) for **2** hours on a rotator set at 100 rpm
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 100 µl of diluted Biotin-Conjugate to all wells
7.	Incubation	Cover with a plastic plate cover and incubate at room temperature (18ºC to 25ºC) for **1** hour on a microplate shaker set at 100 rpm. **(shaking is absolutely necessary for an optimal test performance.)**
8.	Wash	Repeat wash step 5.
9.	Addition	Add 100µl of **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**
11.	Wash	Repeat wash step 8.
12.	Addition	Add 100µl of ready-to-use **TMB Substrate Solution** into all wells
13.	Incubation	Incubate for **30 minutes*** at room temperature. Avoid direct exposure to intense light.
14.	Addition	Add 100µl of **Stop Reagent** 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 630 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_

9. Data Analysis

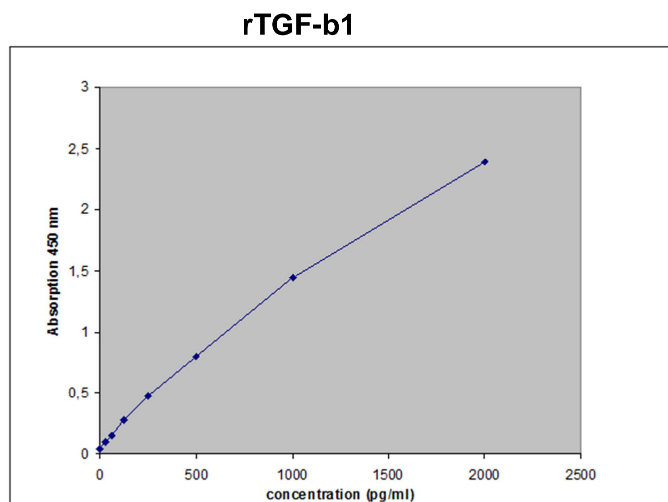
Calculate the average absorbance values for each set of duplicate standards and samples**.** Ideally duplicates should be within 20% of the mean.

Generate a linear standard curve by plotting the average absorbance of each standard on the vertical axis versus the corresponding rTGF-β1 standard concentration on the horizontal axis.

The amount of rTGF-β1 in each sample is determined by extrapolating OD values against rTGF-β1 standard concentrations using the standard curve**.**

Example rTGF-β1 Standard Curve

Standard	rTGF-β1 Conc	O.D. (450nm) mean	C.V. (%)
1	2000	2.390	0.8
2	1000	1.442	4.3
3	500	0.801	1.8
4	250	0.474	2.7
5	125	0.279	2.1
6	62.5	0.154	0.4
Blank	0	0.043	1.6



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.

Note: For samples which have been diluted according to the instructions given in this manual 1:24, the concentration read from the standard curve must be multiplied by the dilution factor (x24)

10. 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.**

11. Performance Characteristics

11.1. Sensitivity

The limit of detection for recombinant rat 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 8 pg/ml (mean of 6 independent assays).

11.2. Specificity

The ELISA was shown to specifically detect rat TGF-β1. No cross reactivity was apparent with serum factors spiked into rat serum, notably there was no cross reactivity with rat TGF-β2.

11.3. Precision

Intra-assay

Intra-assay variability was determined by 6 replicates of 8 serum samples. **The average coefficient of variation was 3.7%.**

Inter-assay

Inter-assay variability was determined by 6replicates of 8 serum samples. **The average coefficient of variation was 8.6%.**

11.4. Dilution Parallelism

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

Linearity of dilution was measured in various samples. For Recovery data see table below:

Sample Matrix	Recovery of Exp. Val.	
	Range (%)	Mean (%)
Serum	78-112	93
Plasma	78-126	105
Cell culture supernatant	90-115	106

11.5. Spike Recovery

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

Sample Matrix	Spike high (%)	Spike medium (%)	Spike low (%)
Serum	88	85	79
Plasma (Heparin)	97	100	97
Cell culture Supernatant	124	141	131

11.6. 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 rat TGF-β1 level determined after 24 h. There was no significant loss of rat TGF-β1 immunoreactivity detected during storage inder above conditions.

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

12. Bibliography

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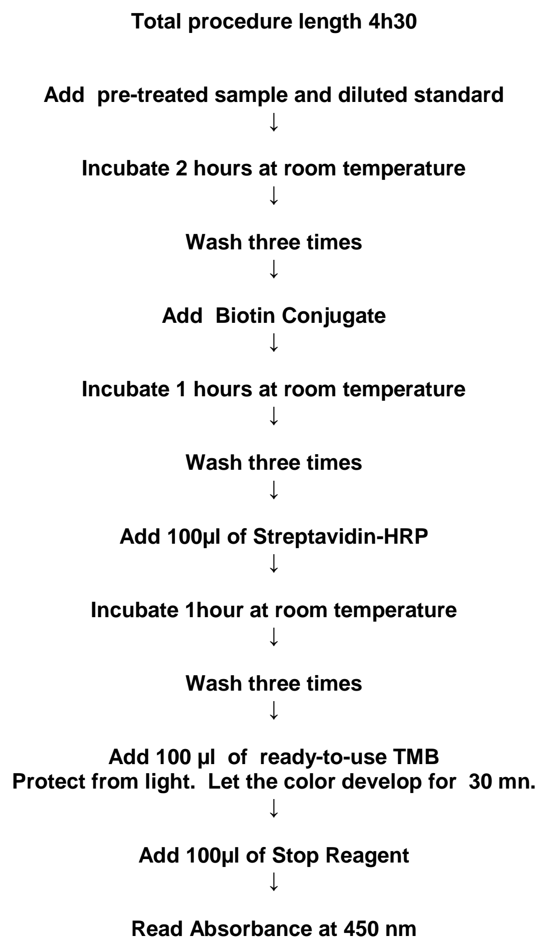
13. Assay Summary



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