

Rat TGF- β 1 ELISA Kit

Instructions for use

Catalogue numbers: 1x96 tests: 670 020 096

For research use only

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Rat TGF- β 1 ELISA Kit

1. Intended use

The rat TGF- β 1 ELISA is an enzyme-linked immunosorbent assay for quantitative detection of rat transforming growth factor beta-1 (rTGF- β 1) in cell culture supernatants, rat serum, plasma or other body fluids.

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 (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 mesenchymal 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- β 1 is the first recognized transforming growth factor (1), its subunits of each 12.5 kDa are bound via disulphide bridges. TGF- β 1 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-rTGF- β 1 coating antibody is adsorbed onto microwells. rTGF- β 1 present in the sample or standard binds to antibodies adsorbed to the microwells. Following incubation unbound biological components are removed during a wash step. A biotin conjugated anti-rTGF- β 1 antibody is added and binds to rTGF- β 1 captured by the first antibody.

Following incubation unbound biotin conjugated anti-rTGF- β 1 is removed during a wash step. Streptavidin-HRP is added and binds to the biotin conjugated anti-rTGF- β 1. 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- β 1 present in the sample or standard. The reaction is terminated by addition of acid and absorbance is measured at 450nm. A standard curve is prepared from rTGF- β 1 standard dilutions and rTGF- β 1 sample concentration determined.

3. Reagents provided and reconstitution

REAGENTS (store at 2-8°C)	Quantity 1x96 well kit 670.020.096	RECONSTITUTION
96-well precoated microtiter plate	1	Ready-to-use
Plate covers	2	
Biotin-Conjugate anti-rat TGF-β1 antibody	1 vial	Dilute 100 times in Assay Buffer (120µl)
Streptavidin-HRP	1 vial	Dilute 200 times in Assay Buffer (150µl)
Rat TGF-β1 Standard	2 vials	Reconstitute as indicated on the vial
Assay Buffer Concentrate	2 vials	(5 ml) 20X concentrate. Dilute in distilled water
Wash Buffer Concentrate	1 vial	(50 ml) 20X concentrate. Dilute in distilled water
Substrate Solution	1 vial	(15 ml) Ready-to-use
Stop Solution (1M Phosphoric acid)	1 vial	(15 ml) Ready-to-use

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. 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 30 days.

Assay Buffer 1X: Once prepared, store at 2-8°C for up to 30 days.

6. 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.

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 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 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 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

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 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	0	0										
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°C to 8°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 rTGF- β 1. **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 100 μ l of Assay buffer to all standard and blank wells
- Immediately after reconstitution add 100 μ l of the reconstituted standard to well's 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 100 μ l 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 100 μ l from wells B1 and B2 through to wells F1 and F2 providing a serial diluted standard curve ranging from 2000 to 62.5 pg/ml
- Discard 100 μ l 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.

a. Serum and plasma samples

Dilute serum and plasma with Assay Buffer 1X : 920 μ l Assay Buffer 1X + 20 μ l sample.

Add 30 μ l 1N HCl to 940 μ l prediluted sample

Mix and incubate for 1 hour at room temperature. Neutralize by addition of 30 μ l 1N NaOH.

It is absolutely necessary to vortex the samples.

b. Cell culture supernatant samples

Dilute cell culture supernatant with Assay Buffer 1X : 180 μ l Assay Buffer 1X + 20 μ l sample.

Add 20 μ l 1N HCl to 200 μ l prediluted sample

Mix and incubate for 1 hour at room temperature. Neutralize by addition of 20 μ l 1N NaOH.

It is absolutely necessary to vortex the samples.

8.6. Preparation of Biotin Conjugate

Please note that the biotin-conjugate should be used within 30 minutes after dilution.

Make a 1:100 dilution with Assay Buffer (1X) in a clean plastic tube as needed according to the following table:

Number of Strips	Biotin-Conjugate (μ l)	Assay Buffer (ml)
1 - 6	60	5.94
1 - 12	120	11.88

8.7. 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 (μ l)	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 except the standard vial which must be mixed gently by inversion only.

Prepare all reagents and pretreat samples 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
2.	Addition	Prepare Standard curve as shown in section 8.4 above
3.	Addition	In blank wells : add 100µl of Assay Buffer 1X in duplicate In sample wells in duplicate : <u>For serum and plasma</u> : add 80 µl of Assay buffer 1X + 20 µl of each pretreated samples (see section 8.5) <u>For cell culture supernatant</u> add 60 µl of Assay buffer 1X + 40 µl of each pretreated samples (see section 8.5)
4.	Incubation	Cover with a plastic plate cover and incubate at room temperature (18°C to 25°C) for 2 hours on a rotator set at 100 rpm (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 100 µl of diluted Biotin-Conjugate to all wells
7.	Incubation	Cover with a plastic plate cover and incubate at room temperature (18°C to 25°C) 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 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 30 minutes on a microplate shaker set at 100 rpm. (shaking is absolutely necessary for an optimal test performance)
11.	Wash	Repeat wash step 5.
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 light by wrapping the plate in aluminium foil
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

10. Data Analysis

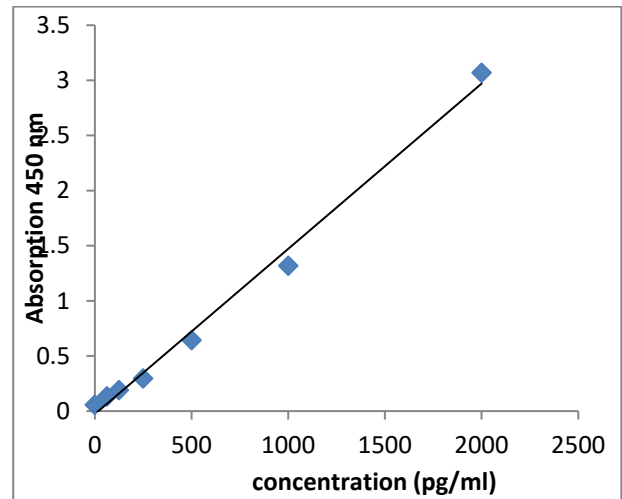
Calculate the average absorbance values for each set of duplicate standards and samples. Ideally duplicates should be within 20% of the mean.

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	OD (450nm) mean	CV (%)
1	2000	3.069	4.1
2	1000	1.319	4.7
3	500	0.644	1.6
4	250	0.297	4.5
6	125	0.191	0
7	62.5	0.134	1.1
Zero	0	0.057	2.5



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 instructions given in this manual :

- Serum and plasma have been diluted 1:250.
- Cell culture supernatant have been diluted 1:30

The concentration read from the standard curve must be multiplied by the dilution factor (x250 or 30, respectively)

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 recombinant rTGF- β 1 defined as the analyte concentration resulting in an absorbance significantly higher than that of the dilution medium (mean plus two standard deviations) was determined to be 7.8 pg/ml (mean of 6 independent assays).

12.2. Specificity

The assay detects both natural and recombinant rat TGF- β 1.

The cross reactivity of TGF- β 2 and TGF- β 3, and of TNF β , IL-8, IL-6, IL-2, TNF α , IL1 β , IL-4, IFN γ , IL12p70, IL-5 and IL-10 was evaluated by spiking these proteins at physiologically relevant concentrations into serum. There was no cross reactivity detected.

12.3. Precision

Intra-assay

Reproducibility within the assay was evaluated in 3 independent experiments. Each assay was carried out with 6 replicates of 4 serum samples containing different concentrations of rTGF- β 1. 2 standard curves were run on each plate. **The calculated overall intra-assay coefficient of variation was 6.8%.**

Inter-assay

Assay to assay reproducibility within one laboratory was evaluated in 3 independent experiments. Each assay was carried out with 6 replicates of 4 plasma samples containing different concentrations of rTGF- β 1. 2 standard curves were run on each plate. **The calculated overall inter-assay coefficient of variation was 6.8%.**

12.4. Dilution Parallelism

Serum, plasma and cell culture supernatant samples with different levels of rTGF- β 1 were analysed at serial 2 fold dilutions with 4 replicates each. Experiments showed an **overall mean recovery of 93%.**

12.5. Spike Recovery

The spike recovery was evaluated by spiking 3 levels of rTGF- β 1 into serum, plasma and cell supernatant. Recoveries were determined with 4 replicates each. Observed values showed an **overall mean recovery of 85%.**

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 rTGF- β 1 level determined after 24 h. There was no significant loss of rTGF- β 1 immunoreactivity detected during storage under above conditions.

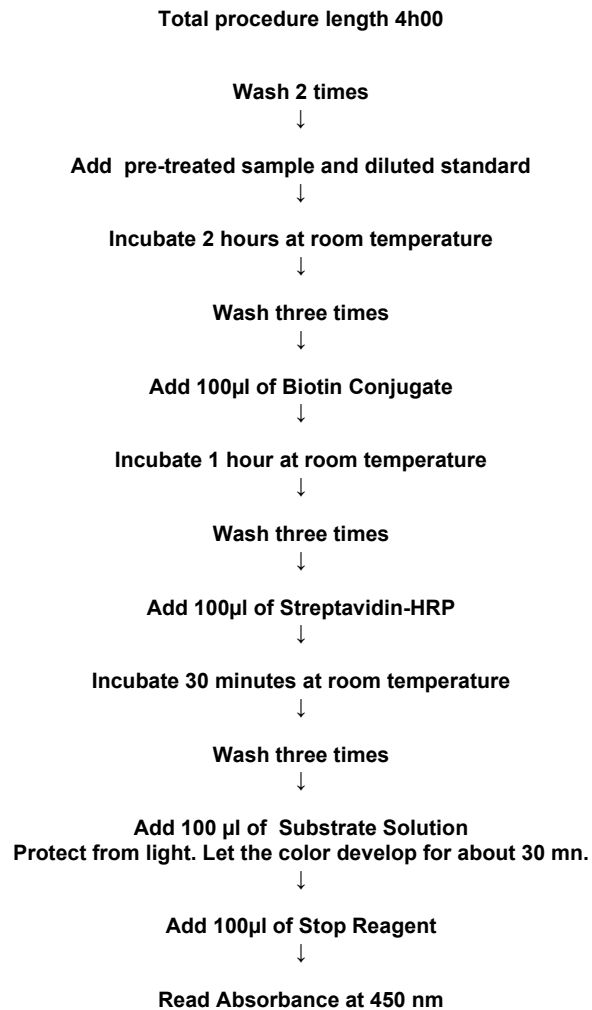
Freeze-thaw Stability

Aliquots of serum samples (unspiked or spiked) were stored at -20°C and thawed up to 5 times, and the rTGF- β 1 levels determined. There was no significant loss of rTGF- β 1 immunoreactivity detected by freezing or thawing.

13. Bibliography

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14. Assay Summary



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