Murine TGF-β1 ELISA Kit

Instructions for use

Catalogue numbers: 1x96 tests: 660.050.096

For research use only

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Murine TGF-β1 ELISA KIT

1. Intended use

The Murine TGF-β1 ELISA is an enzyme-linked immunosorbent assay for the quantitative detection of mouse transforming growth factor beta-1 levels in cell culture supernatants, murine 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 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 (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-mTGF-β1 coating antibody is adsorbed onto microwells.

mTGF-β1 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 monoclonal anti-mTGF-β1 antibody is added and binds to mTGF-β1 captured by the first antibody.

Following incubation unbound biotin conjugated anti-mTGF-β1 is removed during a wash step. Streptavidin-HRP is added and binds to the biotin conjugated anti-mTGF-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 mTGF-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 mTGF-β1 standard dilutions and mTGF-β1 sample concentration determined.

3. Reagents provided and reconstitution

REAGENTS (store at 2-8°C)	Quantity 1x96 well kit 660.050.096	RECONSTITUTION
96-wells precoated microtiter plate	1	Ready-to-use
Plate covers	2	
Biotin-Conjugate anti-mTGF-β1 monoclonal antibody	1 vial	Dilute 100 times in Assay Buffer (120µl)
Streptavidin-HRP	1 vial	Dilute 100 times in Assay Buffer (150µl)
mTGF-β1 Standard	2 vials	See label 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

- 1N NaOH and 1N HCL are needed to run the test
- 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.

Transfer to a clean wash bottle and store at 2° to 25°C. Please note that the Wash Buffer (1x) is stable for 30 days.

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 (1x) is stable for 30 days.

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.

For your serum and plasmas samples: dilute serum, plasma with Assay Buffer (20 µl sample + 920 µl Assay Buffer). Add 30 µl 1N HCl to 940 µl of prediluted sample, mix and incubate for 1 hour at room temperature. Neutralize by addition of 30 µl 1N NaOH. Vortex.

For your cell culture supernatants samples: dilute cell culture supernatants with Assay Buffer (20µl sample + 180µl Assay Buffer). 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. Vortex.

Sample Matrix	Sample Volume (µl)	Assay Buffer 1x (µl)	HCl 1N (µl)	NaOH 1N (µl)	Dilution
Serum and Plasmas	20	920	30	30	1:50
Cell culture Supernatant	20	180	20	20	1:12

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

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
4.	Addition	For **serum and plasma samples**, add 80µl of Assay Buffer to the sample wells. For **cell culture supernatants samples** add 60µl of Assay Buffer to the sample wells.
5.	Addition	For **serum and plasma samples** add 20µl of each pretreated sample in duplicate to the sample wells. For **cell culture supernatants samples** add 40µl of each pretreated sample in duplicate to the sample wells.
6.	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
7.	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
8.	Addition	Add 100 µl of **diluted Biotin-Conjugate** to all wells
9.	Incubation	Cover with a plastic plate cover and incubate at room temperature (18ºC to 25ºC) for **1 hour** on a rotator set at 100 rpm
10.	Wash	Repeat wash step 7.
11.	Addition	Add 100µl of **Streptavidin-HRP** solution into all wells
12.	Incubation	Cover with a plastic plate cover and incubate at room temperature (18ºC to 25ºC) for **30 minutes** on a rotator set at 100 rpm
13.	Wash	Repeat wash step 7.
14.	Addition	Add 100µl of ready-to-use **TMB Substrate Solution** into all wells
15.	Incubation	Incubate for **30 minutes*** at room temperature. Avoid direct exposure to intense light.
16.	Addition	Add 100µl of **Stop Reagent** into all wells
Read the absorbance value of each well (immediately after step 16.) on a spectrophotometer using 450 nm as the primary wavelength and optionally 630 nm as the reference wavelength (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

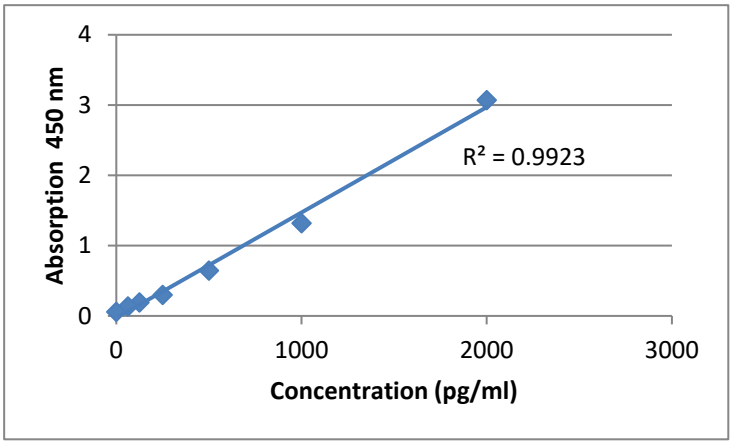
Calculate the average absorbance values for each set of duplicate standards and samples. Ideally duplicates should be within 20% of the mean.

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

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

Example mTGF-β1 Standard Curve

Standard	mTGF-β1 Conc	O.D. (450nm) mean	C.V. (%)
1	2000	3.069	4.1
2	1000	1.3019	4.7
3	500	0.644	1.6
4	250	0.297	4.5
5	125	0.191	0
6	62.5	0.134	1.1
Blank	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.

Note: For samples which have been diluted according to the instructions given in this manual 1:250 serums and plasmas or 1:30 cell culture supernatant the concentration read from the standard curve must be multiplied by the dilution factor (x250, x30 respectively).

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 mTGF-β1, 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 7.8 pg/ml (mean of 6 independent assays).

11.2. Specificity

The ELISA detects both natural and recombinant mTGF-β1. The cross reactivity of TGF-β2 and TGFβ3 and of TNFβ, IL-8, IL-6, IL-2, TNFα, IL-1β, IL-4, IFNγ, IL-12p70, IL-5 and IL-10 was evaluated by spiking these proteins at physiologically releavant concentrations into serum. There was no cross reactivity detected.

11.3. Precision

Intra-assay

Intra-assay variability was determined by 6 replicates of 8 serum samples. **The average coefficient of variation was 7.9%.**

Inter-assay

Inter-assay variability was determined by 6 replicates of 8 serum samples. **The average coefficient of variation was 5.8%.**

11.4. Dilution Parallelism

Serum, plasma and cell culture supernatant samples with different levels of mTGF-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	89-119	103
Plasma (EDTA)	90-115	100
Plasma (citrate)	81-109	94
Cell culture supernatant	-	105

11.5. Spike Recovery

For recovery data see table below:

Sample Matrix	Spike high (%)	Spike medium (%)	Spike low (%)
Serum	85	99	99
Plasmas (EDTA)	81	83	73
Plasmas (citrate)	96	89	97
Cell culture Supernatant	87	85	95

11.6. Stability

Storage Stability
Aliquots of Serum samples (spiked or unspiked) were stored at -20°C, 2-8°C and room temperature (RT) and the mTGF-β1 determined after 24h. There was no significant loss of immunoreactivity detected during storage under above conditions.

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

12. Bibliography

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2. Kropf J, JO Schurek, A Wollner, and AM Gressner. Immunological measurement of transforming growth factor-beta I (TGF-β1) in blood; assay development and comparison.Clinical Chemistry 1997:43(10):1965-1974.

3. Lawrence DA. Transforming growth factor-beta: a general review Eur Cytokine Netw 1996 Sep;7(3):363-374.

13. Assay Summary

Total procedure length : 4h00

Add pre-treated sample and diluted standard
↓
Incubate 2 hours at room temperature
↓
Wash three times
↓
Add Biotin Conjugate
↓
Incubate 1 hour at room temperature
↓
Wash three times
↓
Add 100µl of Streptavidin-HRP
↓
Incubate 30 min at room temperature
↓
Wash three times
↓
Add 100 µl of ready-to-use TMB
Protect from light. Let the color develop for 30 min.
↓
Add 100µl of Stop Reagent
↓
Read Absorbance at 450 nm

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