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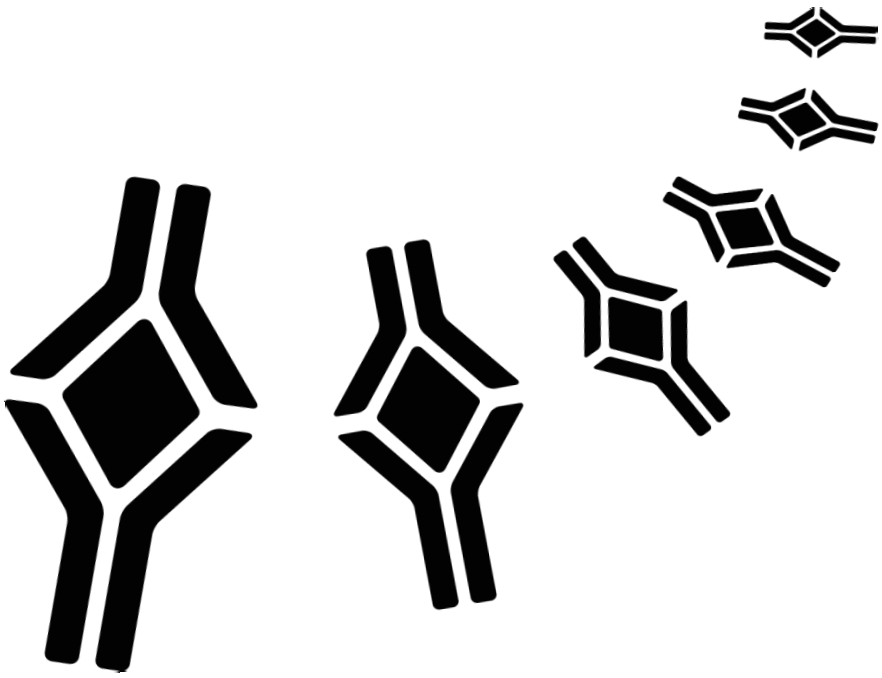


Rat TGF – beta1 ELISA

Product Data Sheet

Cat. No.: RAF124R

For Research Use Only

CONTENTS

» **This kit is manufactured by:**
BioVendor – Laboratorní medicína a.s.

» **Use only the current version of Product Data Sheet enclosed with the kit!**

1. INTENDED USE

The rat TGF-β1 ELISA is an enzyme-linked immunosorbent assay for the quantitative detection of rat TGF-β1. **The rat TGF-β1 ELISA is for research use only. Not for diagnostic or therapeutic procedures.**

2. SUMMARY

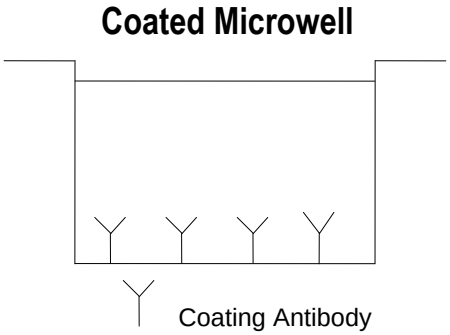
Transforming growth factor-β (TGF-β) is a pleiotropic cytokine that exhibits a broad spectrum of biological and regulatory effects on the cellular and organism level. It plays a critical role in cellular growth, development, differentiation, proliferation, extracellular matrix (ECM) synthesis and degradation, control of mesenchymal-epithelial interactions during embryogenesis, immune modulation, apoptosis, cell cycle progression, angiogenesis, adhesion and migration and leukocyte chemotaxis. It has both tumor suppressive and tumor promoting activities and is highly regulated at all levels (e.g.: mRNA turnover, latent protein activation or post-translational modifications). TGF-β is the first recognized protein of at least 40 of the TGF-β superfamily of structurally related cytokines. Three isoforms (TGF-β 1-3) have been described in mammals. (Each isoform is encoded by a unique gene on different chromosomes. All bind to the same receptors.) They are synthesized by most cell types and tissues. Cells of the immune system mainly express TGF-β 1. The initially sequestered, inactive LTGF-β (latent TGF-β) requires activation (cleavage and dissociation of its LAP (latency associated peptide) region) before it can exert biological activity. LTGF-β can also be bound to LTB (latent TGF-β binding protein) to form a large latent complex (LLC). TGF-β forms homodimers, and its subunits of 12.5 kDa each are bound via disulphide bridges. TGF-β signal transduction is mediated via the TGF-β receptors Type II and I, phosphorylation and conformational changes, followed by different pathways: SMAD (- pathway: TGF-β recruitment finally leads to phosphorylation of receptor-regulated SMADs (R-SMADs = SMAD 2, 3) and binding of common SMAD (coSMAD = SMAD 4).
The R-SMAD/ coSMAD complex enters the nucleus and interacts with a number of transcription factors, coactivators and corepressors. TGF-β induces MAPK- and MAP/ERK kinase dependent signal transduction (JNK/MAPK-, JNK/SPAK-, p38-, ERK1/2 - pathway) and the NF-κB – pathway. TGF-β mediates cell cycle growth arrest via the phosphoinositide 3-kinase/Akt pathway. TGF-β signaling is highly regulated e.g. via interaction with inhibitory SMADs (I-SMADs = SMAD 6, 7) or binding of the E3-ubiquitin ligases Smurf1 and Smurf2 or/and coreceptors. TGF-β1 is the key mediator in the pathophysiology of tissue repair and human fibrogenesis: balance between production and degradation of type I collagen, and fibrosis and scarring in organ and tissue.
TGF-β1 exhibits important immunoregulatory features of partially adverse character: TGF-β1 inhibits B and T cell proliferation, differentiation and antibody production as well as maturation and activation of macrophages.

It inhibits activity of NK cells and lymphokine activated killer cells and blocks production of cytokines. TGF-β1 promotes Treg cell differentiation resulting in IL-10/TGF-β1 production and Th1 cell and Th2 cell suppression.
TGF-β1 was recently shown to promote Th17 development in the presence of IL-6 or IL-21 in mice and probably plays a role in human Th17 development together with IL-1β, IL-21 and IL-23. In this context TGF-β1 is involved in induction and mediation of proinflammatory and allergic responses.

3. PRINCIPLES OF THE TEST

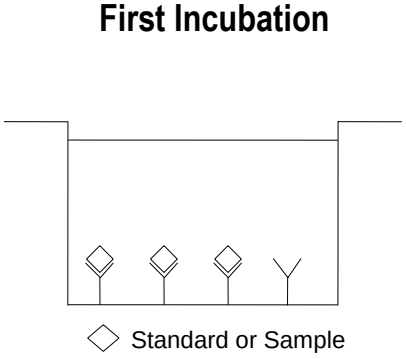
An anti-rat TGF-β1 coating antibody is adsorbed onto microwells.

Figure 1



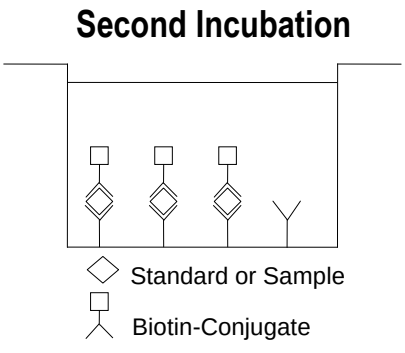
Rat TGF-β1 present in the sample or standard binds to antibodies adsorbed to the microwells.

Figure 2



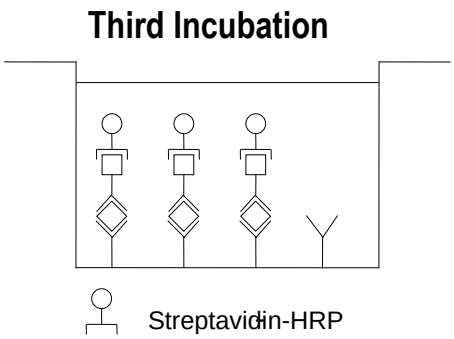
Following incubation unbound biological components are removed during a wah step. A biotin-conjugated anti-rat TGF-β1 antibody is added and binds to rat TGF-β1 captured by the first antibody.

Figure 3



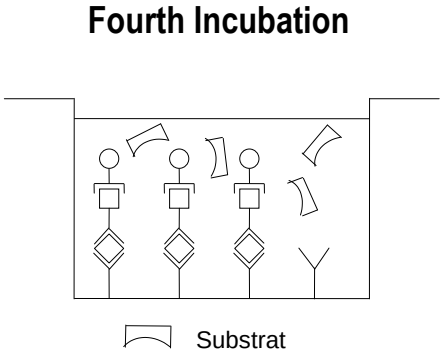
Following incubation unbound biotin-conjugated anti-rat TGF-β1 antibody is removed during a wash step. Streptavidin-HRP is added and binds to the biotin-conjugated anti-rat TGF-β1 antibody.

Figure 4



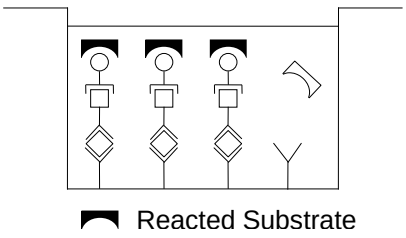
Following incubation unbound Streptavidin-HRP is removed during a wash step, and substrate solution reactive with HRP is added to the wells.

Figure 5



A coloured product is formed in proportion to the amount of rat TGF-β1 present in the sample or standard. The reaction is terminated by addition of acid and absorbance is measured at 450 nm. A standard curve is prepared from 7 rat TGF-β1 standard dilutions and rat TGF-β1 sample concentration determined.

Figure 6



4. REAGENTS PROVIDED

1 aluminium pouch with a **Antibody Coated Microtiter Strips** with monoclonal antibody to rat TGF-β1
1 vial (120 µl) **Biotin-Conjugate** anti-rat TGF-β1 polyclonal antibody
1 vial (150 µl) **Streptavidin-HRP**
2 vials rat TGF-β1 **Standard** lyophilized, 4000 pg/ml upon reconstitution
2 vials (5 ml) **Assay Buffer Concentrate** 20x
(PBS with 1% Tween 20 and 10% BSA)
1 bottle (50 ml) **Wash Buffer Concentrate** 20x
(PBS with 1% Tween 20)
1 vial (15 ml) **Substrate Solution** (tetramethyl-benzidine)
1 vial (15 ml) **Stop Solution** (1M Phosphoric acid)
6 **Adhesive Films**

5. STORAGE INSTRUCTIONS – ELISA KIT

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 labels. 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, this reagent is not contaminated by the first handling.

6. SPECIMEN COLLECTION AND STORAGE INSTRUCTIONS

Cell culture supernatant *, serum and plasma (EDTA, citrate) were tested with this assay. Other biological samples might be suitable for use in the assay. Remove serum or plasma from the clot or cells as soon as possible after clotting and separation.
Samples containing a visible precipitate must be clarified prior to use in the assay. Do not use grossly hemolyzed or lipemic specimens.
Samples should be aliquoted and must be stored frozen at -20°C to avoid loss of bioactive rat TGF-β1. If samples are to be run within 24 hours, they may be stored at 2° to 8°C (for sample stability refer to 13.5).
Avoid repeated freeze-thaw cycles. Prior to assay, the frozen sample should be brought to room temperature slowly and mixed gently.
* Pay attention to a possibly elevated blank signal in cell culture supernatant samples containing serum components (e.g. FCS), due to latent TGF--β levels in animal serum.

7. MATERIALS REQUIRED BUT NOT PROVIDED

- 1N NaOH and 1N NCL are needed to run the test
- 5 ml and 10 ml graduated pipettes
- 5 µl to 1000 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)
- Microplate shaker
- 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 regression analysis

8. PRECAUTIONS FOR USE

- All chemicals should be considered as potentially hazardous. We therefore recommend that this product is handled only by those persons who have been trained in laboratory techniques and that it is used in accordance with the principles of good laboratory practice. Wear suitable protective clothing such as laboratory overalls, safety glasses and gloves. Care should be taken to avoid contact with skin or eyes. In the case of contact with skin or eyes wash immediately with water. See material safety data sheet(s) and/or safety statement(s) for specific advice.
- 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.
- Avoid contact of substrate solution 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 reagent.
- Exposure to acid inactivates the conjugate.
- Glass-distilled water or deionized water must be used for reagent preparation.
- Substrate solution must be at room temperature prior to use.
- Decontaminate and dispose specimens and all potentially contaminated materials as 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.

9. PREPARATION OF REAGENTS

Buffer Concentrates should be brought to room temperature and should be diluted before starting the test procedure.
If crystals have formed in the **Buffer Concentrates**, warm them gently until they have completely dissolved.

9.1 Wash Buffer (1x)

Pour entire contents (50 ml) of the **Wash Buffer Concentrate** (20x) into a clean 1000 ml graduated cylinder. Bring to final volume of 1000 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 Wash Buffer (1x) is stable for 30 days.

Wash Buffer (1x) may also be prepared as needed according to the following table:

Number of Strips	Wash Buffer Concentrate (20x) (ml)	Distilled Water (ml)
1 - 6	25	475
1 - 12	50	950

9.2 Assay Buffer (1x)

Pour the entire contents (5 ml) of the **Assay Buffer Concentrate** (20x) into a clean 100 ml graduated cylinder. Bring to final volume of 100 ml with distilled water. Mix gently to avoid foaming.
Store at 2° to 8°C. Please note that the Assay Buffer (1x) is stable for 30 days.

Assay Buffer (1x) may also be prepared as needed according to the following table:

Number of Strips	Assay Buffer Concentrate (20x) (ml)	Distilled Water (ml)
1 - 6	2.5	47.5
1 - 12	5.0	95.0

9.3 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 (1x) in a clean plastic tube as needed according to the following table:

Number of Strips	Biotin-Conjugate (ml)	Assay Buffer (1x) (ml)
1 - 6	0.06	5.94
1 - 12	0.12	11.88

9.4 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 (1x) in a clean plastic tube as needed according to the following table:

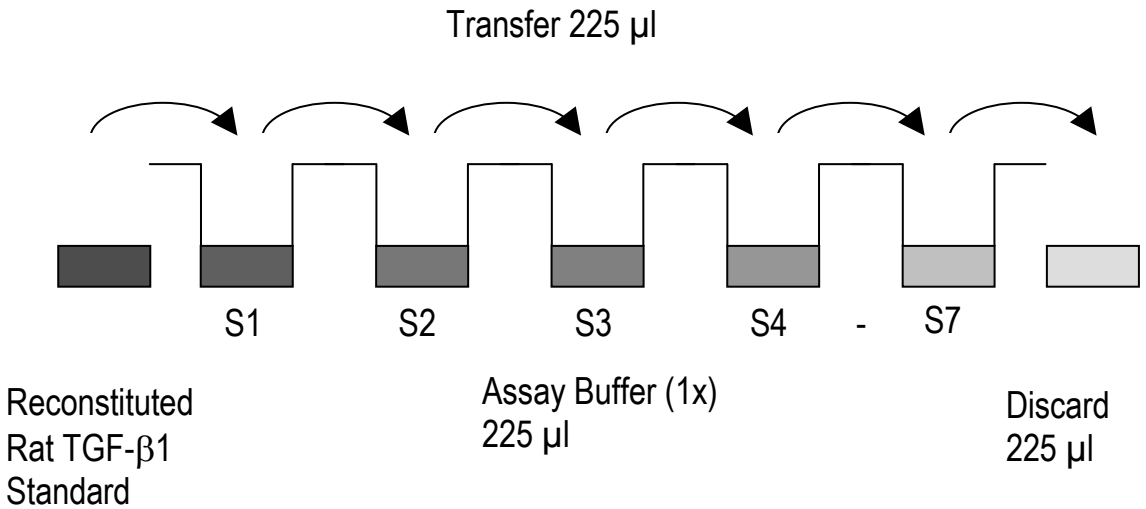
Number of Strips	Streptavidin-HRP (ml)	Assay Buffer (1x) (ml)
1 - 6	0.06	5.94
1 - 12	0.12	11.88

9.5 Rat TGF-β1 Standard

Reconstitute **rat TGF-β1 standard** by addition of distilled water.
Dilution volume is stated in the Quality Control Sheet. Swirl or mix gently to insure complete and homogeneous solubilization
(concentration of reconstituted standard = 4000 pg/ml).
Allow the standard to reconstitute for 10-30 minutes. Mix well prior to making dilutions.
After usage remaining standard cannot be stored and has to be discarded.
Standard dilutions can be prepared directly on the microwell plate (see 10.d) or alternatively in tubes (see 9.5.1).

9.5.1 External Standard Dilution

Label 7 tubes, one for each standard point.
S1, S2, S3, S4, S5, S6, S7
Then prepare 1:2 serial dilutions for the standard curve as follows:
Pipette 225 µl of Assay Buffer (1x) into each tube.
Pipette 225 µl of reconstituted standard (concentration of standard = 4000 pg/ml) into the first tube, labelled S1, and mix (concentration of standard 1 = 2000 pg/ml).
Pipette 225 µl of this dilution into the second tube, labelled S2, and mix thoroughly before the next transfer. Repeat serial dilutions 5 more times thus creating the points of the standard curve (see Figure 7).
Assay Buffer (1x) serves as blank
.Figure 7



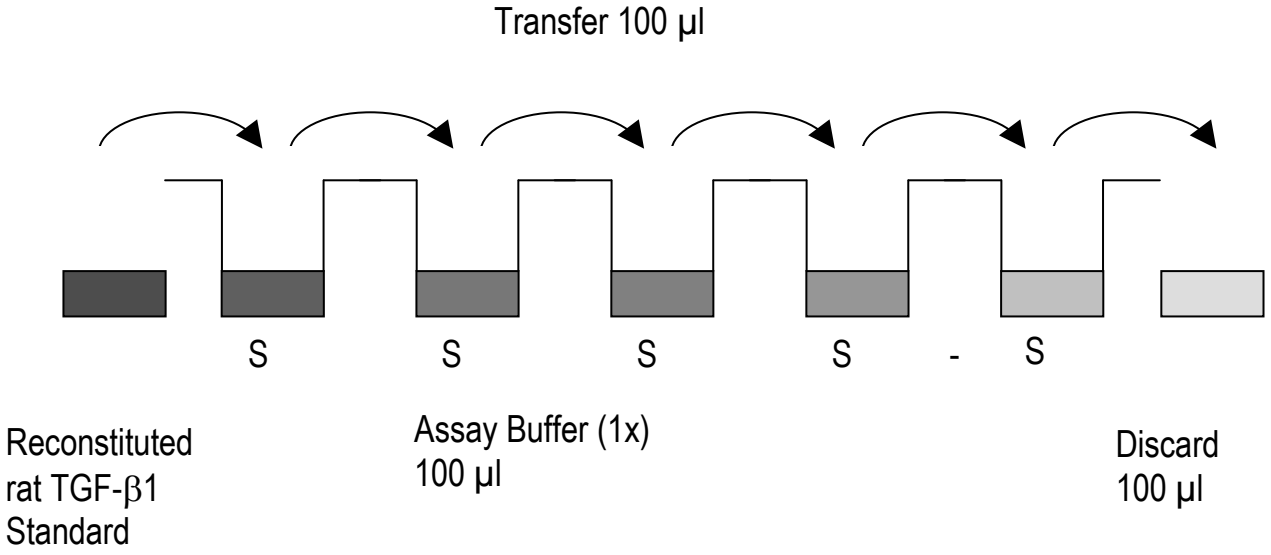
10. TEST PROTOCOL

a. Prepare your serum and plasma samples before starting the test procedure. Dilute serum and plasma samples with Assay Buffer (1x) according to the following scheme:
20 µl sample + 920 µl Assay Buffer (1x)
Add 30 µl 1N HCl (see 7) to 940 µl prediluted sample, mix and incubate for 1 hour at room temperature.
Neutralize by addition of 30 µl 1N NaOH (see 7). **Vortex!**
Prepare your cell culture supernatant samples before starting the test procedure. Dilute cell culture supernatant samples with Assay Buffer (1x) according to the following scheme:
20 µl sample + 180 µl Assay Buffer (1x)
Add 20 µl 1N HCl (see 7) to 200 µl prediluted sample, mix and incubate for 1 hour at room temperature. Neutralize by addition of 20 µl 1N NaOH (see 7). **Vortex!**

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

b. 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 extra microwell strips from holder and store in foil bag with the desiccant provided at 2°-8°C sealed tightly.
c. Wash the microwell strips twice with approximately 400 µl **Wash Buffer** per well with thorough aspiration of microwell contents between washes. Allow the Wash Buffer to sit in the wells for about **10 – 15 seconds** before aspiration. Take care not to scratch the surface of the microwells. After the last wash step, empty wells and tap microwell strips on absorbent pad or paper towel to remove excess Wash Buffer. Use the microwell strips immediately after washing. Alternatively microwell strips can be placed upside down on a wet absorbent paper for not longer than 15 minutes. **Do not allow wells to dry**.
d. **Standard dilution on the microwell plate** (Alternatively the standard dilution can be prepared in tubes – see 9.5.1): Add 100 µl of Assay Buffer (1x) in duplicate to all **standard wells**. Pipette 100 µl of prepared **standard** (see Preparation of Standard 0, concentration = 4000.0 pg/ml) in duplicate into well A1 and A2 (see Table 1). Mix the contents of wells A1 and A2 by repeated aspiration and ejection (concentration of standard 1, S1 = 2000.0 pg/ml), and transfer 100 µl to wells B1 and B2, respectively (see Figure 8). Take care not to scratch the inner surface of the microwells. Continue this procedure 5 times, creating two rows of rat TGF-β1 standard dilutions ranging from 2000.0 to 31.3 pg/ml. Discard 100 µl of the contents from the last microwells (G1, G2) used.

Figure 8



In case of an **external standard dilution** (see 0), pipette 100 µl of these standard dilutions (S1 - S7) in the standard wells according to Table 1.

Table 1
Table depicting an example of the arrangement of blanks, standards and samples in the microwell strips:

	1	**2**	**3**	**4**
A	Standard 1 (2000.0 pg/ml)	Standard 1 (2000.0 pg/ml)	Sample 1	Sample 1
B	Standard 2 (1000.0 pg/ml)	Standard 2 (1000.0 pg/ml)	Sample 2	Sample 2
C	Standard 3 (500.0 pg/ml)	Standard 3 (500.0 pg/ml)	Sample 3	Sample 3
D	Standard 4 (250.0 pg/ml)	Standard 4 (250.0 pg/ml)	Sample 4	Sample 4
E	Standard 5 (125.0 pg/ml)	Standard 5 (125.0 pg/ml)	Sample 5	Sample 5
F	Standard 6 (62.5 pg/ml)	Standard 6 (62.5 pg/ml)	Sample 6	Sample 6
G	Standard 7 (31.3 pg/ml)	Standard 7 (31.3 pg/ml)	Sample 7	Sample 7
H	Blank	Blank	Sample 8	Sample 8

e. Add 100 µl of **Assay Buffer (1x)** in duplicate to the **blank wells**.
f. For serum and plasma samples add 80 µl of **Assay Buffer (1x)** to the **sample wells**.
For cell culture supernatant samples add 60 µl of **Assay Buffer (1x)** to the **sample wells. (It is absolutely necessary to vortex the samples!)**
g. For serum and plasma samples add 20 µl of each pretreated **sample** in duplicate to the **sample wells**. For cell culture supernatant samples add 40 µl of each pretreated **sample** in duplicate to the **sample wells. (It is absolutely necessary to vortex the samples!)**
h. Cover with an adhesive film and incubate at room temperature (18 to 25°C) for 2 hours, on a microplate shaker set at 100 rpm. **(Shaking is absolutely necessary for an optimal test performance.)**
i. Prepare **Biotin-Conjugate** (see Preparation of Biotin-Conjugate 0).
j. Remove adhesive film and empty wells. **Wash** microwell strips 5 times according to point c. of the test protocol. Proceed immediately to the next step.
k. Add 100 µl of **Biotin-Conjugate** to all wells.
l. Cover with an adhesive film and incubate at room temperature (18 to 25°C) for 1 hour, on a microplate shaker set at 100 rpm. **(Shaking is absolutely necessary for an optimal test performance.)**
m. Prepare **Streptavidin-HRP** (refer to Preparation of Streptavidin-HRP 0).
n. Remove adhesive film and empty wells. **Wash** microwell strips 5 times according to point c. of the test protocol. Proceed immediately to the next step.
o. Add 100 µl of diluted **Streptavidin-HRP** to all wells, including the blank wells.
p. Cover with an adhesive film and incubate at room temperature (18° to 25°C) for 1 hour, on a microplate shaker set at 100 rpm. **(Shaking is absolutely necessary for an optimal test performance.)**
q. Remove adhesive film and empty wells. **Wash** microwell strips 5 times according to point c. of the test protocol. Proceed immediately to the next step.
r. Pipette 100 µl of **TMB Substrate Solution** to all wells.
s. Incubate the microwell strips at room temperature (18° to 25°C) for **about 30 min**. Avoid direct exposure to intense light. **The colour development on the plate should be monitored and the substrate reaction stopped (see next point of this protocol) before positive wells are no longer properly recordable. Determinatio of the ideal time period for colour development has to be done individually for each assay.** It is recommended to add the stop solution when the highest standard has developed a dark blue colour. Alternatively the colour development can be monitored by the ELISA reader at 620 nm. The substrate reaction should be stopped as soon as Standard 1 has reached an OD of 0.9 – 0.95.
t. Stop the enzyme reaction by quickly pipetting 100 µl of **Stop Solution** into each well. 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.

u. 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 standards.

11. 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 value.
- Create a standard curve by plotting the mean absorbance for each standard concentration on the ordinate against the rat TGF-β1 concentration on the abscissa. Draw a best fit curve through the points of the graph (a 5-parameter curve fit is recommended).
- To determine the concentration of circulating rat 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 rat TGF-β1 concentration.
- **If instructions in this protocol have been followed serum and plasma samples have been diluted 1:250 (20 µl sample + 920 µl Assay Buffer (1x) (= 1:50) + 30µl 1N HCl + 30µl 1N NaOH and 20 µl pretreated sample + 80 µl Assay Buffer (1x) (= 1:5)) and cell culture supernatant samples have been diluted 1:30 (20 µl sample + 180 µl Assay Buffer (1x) + 20µl 1N HCl + 20µl 1N NaOH (= 1:12) and 40 µl pretreated sample + 60 µl Assay Buffer (1x) (= 1:2.5)), the concentration read from the standard curve must be multiplied by the dilution factor (x 250 or 30, respectively).**
- **Calculation of samples with a concentration exceeding standard 1 may result in incorrect, low rat TGF-β1 levels. Such samples require further external predilution according to expected rat TGF-β1 values with Assay Buffer (1x) in order to precisely quantitate the actual rat TGF-β1 level.**
- It is suggested that each testing facility establishes a control sample of known rat TGF-β1 concentration and runs this additional control with each assay. If the values obtained are not within the expected range of the control, the assay results may be invalid.
- A representative standard curve is shown in Figure 9. This curve cannot be used to derive test results. Each laboratory must prepare a standard curve for each group of microwell strips assayed.

Figure 9
Representative standard curve for rat TGF-β1 ELISA. Rat TGF-β1 was diluted in serial 2-fold steps in Assay Buffer (1x). Do not use this standard curve to derive test results. A standard curve must be run for each group of microwell strips assayed.

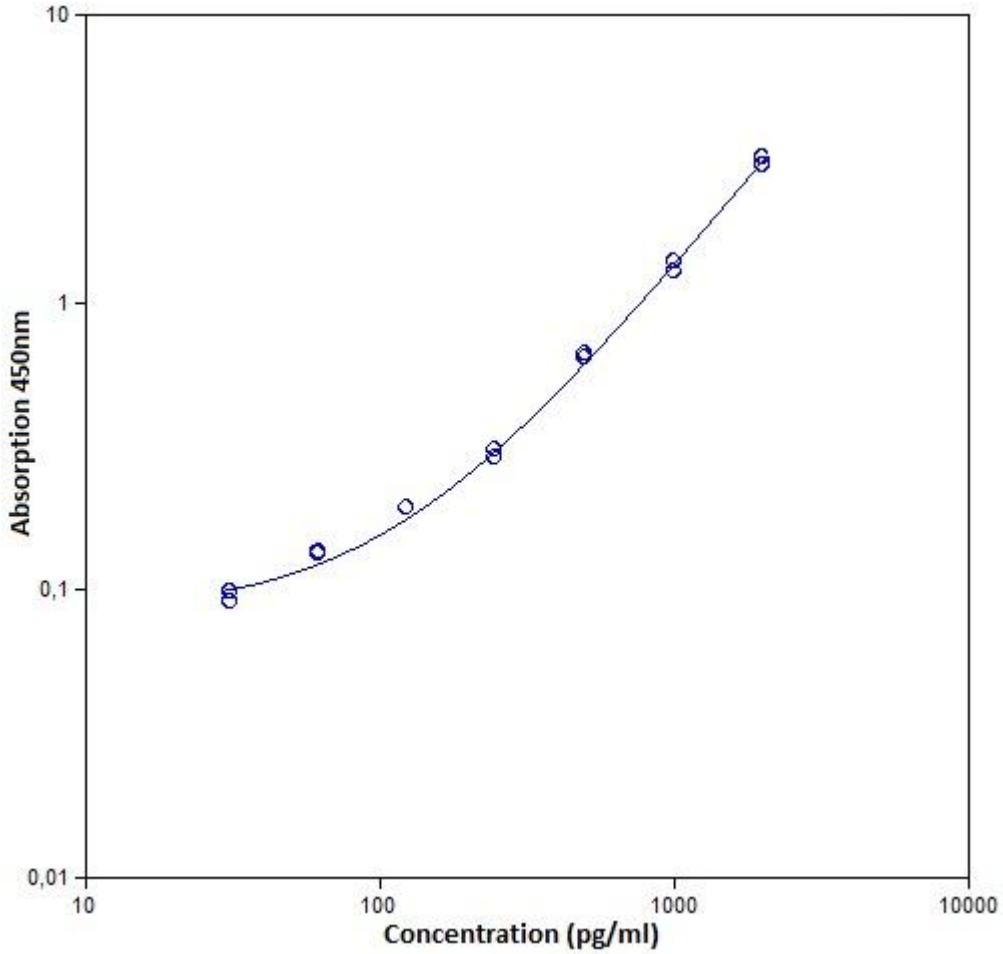


Table 2
Typical data using the rat TGF-β1 ELISA
Measuring wavelength: 450 nm
Reference wavelength: 620 nm

Standard	Rat TGF-β1 Concentration (pg/ml)	O.D. at 450 nm	Mean O.D. at 450 nm	C.V. (%)
1	2000.0	2.979 3.178	3.069	4.1
2	1000.0	1.363 1.257	1.319	4.7
3	500.0	0.636 0.651	0.644	1.6
4	250.0	0.306 0.287	0.297	4.5
5	125.0	0.191 0.191	0.191	0
6	62.5	0.133 0.135	0.134	1.1
7	31.25	0.09 0.098	0.094	6.0
Blank	0	0.056 0.058	0.057	2.5

The OD values of the standard curve may vary according to the conditions of assay performance (e.g. operator, pipetting technique, washing technique or temperature effects). Furthermore shelf life of the kit may affect enzymatic activity and thus colour intensity. Values measured are still valid.

12. LIMITATIONS

- Since exact conditions may vary from assay to assay, a standard curve must be established for every run.
- Bacterial or fungal contamination of either screen 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. Empty wells completely before dispensing fresh wash solution, fill with Wash Buffer as indicated for each wash cycle and do not allow wells to sit uncovered or dry for extended periods.

13. PERFORMANCE CHARACTERISTICS

13.1 Sensitivity

The limit of detection of rat TGF-β1 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 7.8 pg/ml (mean of 6 independent assays).

13.2 Reproducibility

13.2.1 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 rat TGF - β1. 2 standard curves were run on each plate. Data below show the mean rat TGF-β1 concentration and the coefficient of variation for each sample (see Table 3). The calculated overall intra-assay coefficient of variation was 6,8 %

Table 3
The mean rat TGF-β1 concentration and the coefficient of variation for each sample

Sample	Experiment	Mean Rat TGF-β1 Concentration (pg/ml)	Coefficient of Variation (%)
1	1	37500	9.5%
	2	32953	4.5%
	3	31091	7.7%
2	1	28606	7.3%
	2	27682	8.7%
	3	21552	7.8%
3	1	80060	5.9%
	2	80627	5.2%
	3	85132	3.1%
4	1	96306	3.0%
	2	94578	2.0%
	3	84865	16.5%

13.2.2 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 serum plasma samples containing different concentrations of rat TGF-β1. 2 standard curves were run on each plate. Data below show the mean rat TGF-β1 concentration and the coefficient of variation calculated on 18 determinations of each sample (see Table 4). The calculated overall inter-assay coefficient of variation was 6,8 %.

Table 4
The mean rat TGF-β1 concentration and the coefficient of variation of each sample

Sample	Mean Rat TGF-β1 Concentration (pg/ml)	Coefficient of Variation (%)
1	33848	7.3%
2	25947	7.9%
3	81940	4.8%
4	91916	7.2%

13.3 Spiking Recovery

The spiking 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.
For recovery data see Table 5.

Table 5

Sample matrix	Spike high (%)	Spike medium (%)	Spike low (%)
Serum	98	97	112
Plasma (Heparin)	77	81	71
Plasma (EDTA)	72	78	71
Cell culture supernatant	87	85	95

13.4 Dilution Parallelism

Serum, plasma and cell culture supernatant samples with different levels of rat TGF-β1 were analysed at serial 2 fold dilutions with 4 replicates each. For recovery data see Table 6.

Table 6

Sample matrix	Recovery of Exp. Val. Range (%)	Mean (%)
Serum	95 - 106	100
Plasma (Heparin)	76 – 92	85
Plasma (EDTA)	69 – 92	82
Cell culture supernatant	95 - 118	105

13.5 Sample Stability

13.5.1 Freeze-Thaw Stability

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

13.5.2 Storage Stability

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

13.6 Specificity

The assay detects both natural and recombinant rat TGF-β1.
The cross reactivity of TGF-β2 and TGF-β3, and of TNF-β, IL-8, IL-6, IL-2, TNF-α, IL-1β, 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.

14. REAGENT PREPARATION SUMMARY

14.1 Wash Buffer (1x)

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

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

14.2 Assay Buffer (1x)

Add **Assay Buffer Concentrate** 20x (5 ml) to 95 ml distilled water.

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

14.3 Biotin-Conjugate

Make a 1:100 dilution of **Biotin-Conjugate** in Assay Buffer (1x):

Number of Strips	Biotin-Conjugate (ml)	Assay Buffer (1x) (ml)
1 - 6	0.06	5.94
1 - 12	0.12	11.88

14.4 Streptavidin-HRP

Make a 1:100 dilution of **Streptavidin-HRP** in Assay Buffer (1x):

Number of Strips	Streptavidin-HRP (ml)	Assay Buffer (1x) (ml)
1 - 6	0.06	5.94
1 - 12	0.12	11.88

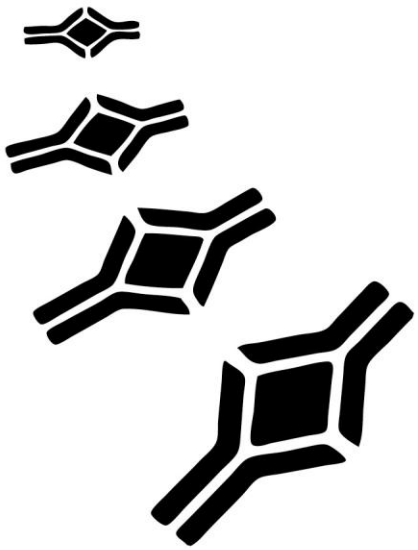
14.5 Rat TGF-β1 Standard

Reconstitute lyophilized **rat TGF-β1 standard** with distilled water. (Reconstitution volume is stated in the Quality Control Sheet.).

15. TEST PROTOCOL SUMMARY

1. Pretreatement for serum and plasma samples: 20 µl sample + 920 µl Assay Buffer (1x), add 30 µl 1N HCl (see 7) to 940 µl prediluted sample, mix and incubate for 1 hour at room temperature, add 30 µl 1N NaOH (see 7) (**Vortex!)**; Pretreatement for cell culture supernatant samples: 20 µl sample + 180 µl Assay Buffer (1x), add 20 µl 1N HCl (see 7) to 200 µl prediluted sample, mix and incubate for 1 hour at room temperature, add 20 µl 1N NaOH (see 7) **(Vortex!)**;
2. Determine the number of microwell strips required.
3. Wash microwell strips twice with Wash Buffer.
4. Standard dilution on the microwell plate: Add 100 µl Assay Buffer (1x), in duplicate, to all standard wells. Pipette 100 µl prepared standard into the first wells and create standard dilutions by transferring 100 µl from well to well. Discard 100 µl from the last wells. Alternatively external standard dilution in tubes (see 0): Pipette 100 µl of these standard dilutions in the microwell strips.
5. Add 100 µl Assay Buffer (1x), in duplicate, to the blank wells.
6. Add 80 µl (serum and plasma samples) or 60 µl (cell culture supernatant samples) Assay Buffer (1x) to sample wells.
7. Add 20 µl (serum or plasma sample) or 40 µl (cell culture supernatant sample) in duplicate, to designated sample wells. **(It is absolutely necessary to vortex the samples!)**
8. Cover microwell strips and incubate 2 hours at room temperature **(Shaking is absolutely necessary for an optimal test performance.)**
9. Prepare Biotin-Conjugate.
10. Empty and wash microwell strips 5 times with Wash Buffer.
11. Add 100 µl Biotin-Conjugate to all wells.
12. Cover microwell strips and incubate 1 hours at room temperature. **(Shaking is absolutely necessary for an optimal test performance.)**
13. Prepare Streptavidin-HRP.
14. Empty and wash microwell strips 5 times with Wash Buffer.
15. Add 100 µl diluted Streptavidin-HRP to all wells.
16. Cover microwell strips and incubate 1 hour at room temperature. **(Shaking is absolutely necessary for an optimal test performance.)**
17. Empty and wash microwell strips 5 times with Wash Buffer.
18. Add 100 µl of TMB Substrate Solution to all wells.
19. Incubate the microwell strips for **about 30 minutes** at room temperature
20. Add 100 µl Stop Solution to all wells.
21. Blank microwell reader and measure colour intensity at 450 nm.

Note: If instructions in this protocol have been followed samples have been diluted 1:250 (serum and plasma) or 1:30 (cell culture supernatant), the concentration read from the standard curve must be multiplied by the dilution factor (x 250 or 30, respectively).



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