DIACLONE
A Tepnel Company

Mouse TGF-β1 ELISA

660 050 096 **1 x 96 tests**
660 050 192 **2 x 96 tests**

INTENDED USE

The mTGF-β1 ELISA is an enzyme-linked immunosorbent assay for the quantitative detection of mouse transforming growth factor beta-1 levels in cell culture supernatants, serum, plasma, or other body fluids.
The mTGF-β1 ELISA is for research use only. Not for use in diagnostic or therapeutic procedures.

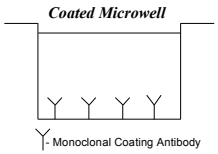
SUMMARY

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).

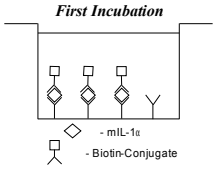
TGF-β1 is the first recognized transforming growth factor (1), its subunits of each 12.5 kDa are bound via disulphide bridges. TGF-β1 is inhibitive to T- and B cell proliferation as well as to maturation and activation of macrophages. It furthermore inhibits activity of natural killer cells and lymphokine activated killer cells and blocks production of cytokines.

PRINCIPLES OF THE METHOD

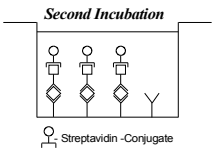
An anti-mTGF-β1 coating antibody is adsorbed onto microwells.



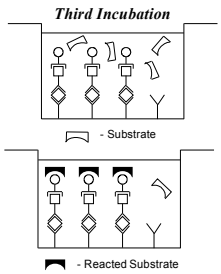
mTGFβ1 present in the sample or standard binds to antibodies adsorbed to the microwells; a biotin-conjugated monoclonal anti-mTGFβ1 antibody is added and binds to mTGFβ1 captured by the first antibody.



Following incubation unbound biotin conjugated anti-mTGFβ1 is removed during a wash step. Streptavidin-HRP is added and binds to the biotin conjugated anti-mTGFβ1 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 mTGFβ1 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 mTGFβ1 standard dilutions and mTGFβ1 sample concentration determined.



REAGENTS PROVIDED

REAGENTS (store at 2-8°C)	QTY 1 plate	QTY 1 plates	RECONSTITUTION
96-wells precoated microtiter plate	1	2	Ready-to-use
Plate covers	2	4	
mTGF-β1 Standard	2 vials	4 vials	See label on the vial
Biotin conjugate monoclonal antibody to mTGF-β1	1 vial	2 vials	120µl
Streptavidin-HRP	1 vial	2 vials	150µl
Assay Buffer Concentrate	2 vial	4 vials	(5 ml) 20X concentrate. Dilute in distilled water
Wash Buffer Concentrate	1 bottle	2 bottles	(50 ml) 20X concentrate. Dilute in distilled water
Substrate Solution	1 vial	2 vials	(15 ml) Ready-to-use
Stop Solution (1 M 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
Red 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

MATERIALS REQUIRED BUT NOT PROVIDED

- 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)
- 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 linear regression analysis

SAFETY

- 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.
- Reagents containing thimerosal as preservative may be toxic if ingested.
- Avoid contact of substrate solutions 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 reagents.
- Exposure to acids will inactivate the conjugate.
- Glass-distilled water or deionized water must be used for reagent preparation.
- Substrate solutions must be at room temperature prior to use.
- Decontaminate and dispose specimens and all potentially contaminated materials as if 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.

STORAGE INSTRUCTIONS

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. 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.

SPECIMEN COLLECTION, PROCESSING AND STORAGE

Cell culture supernatants, murine serum and 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.

Samples containing a visible precipitate must be clarified prior to use in the assay. Do not use grossly haemolysed or lipemic specimens.

Clinical samples should be kept at 2° to 8°C and separated rapidly (within half an hour) before storing at -20°C to avoid loss of bioactivity. Avoid repeated freeze-thaw cycles.

PREPARATION OF REAGENTS

1. 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 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

2. 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 2º to 8ºC. 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

3. Preparation of Biotin Conjugate

Make a 1:100 dilution of the concentrated **Biotin Conjugate** with **Assay Buffer** in a clean plastic test tube. Please note that the Biotin -Conjugate should be used within 30 minutes after dilution. Biotin-Conjugate may be prepared as needed according to the following table:

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

4. Preparation of Streptavidin-HRP

Please Note that the Strepatvidin-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 tubes as needed according to the following table :

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

4. Preparation of Standard

Reconstitute mTGFβ1 **Standard** by addition of **distilled water**. Dilution volume is stated on the label of the standard vial. Mix gently to insure complete homogeneity. (concentration of the reconstituted standard = 4ng/ml)
Allow the standard to reconstitute to 10 minutes. Mix well prior to making dilution.
After usage remaining standard cannot be stored and has to be discarded.
Standards dilutions can be prepared directly on the microwell plate, or alternatively in tubes.

5. Addition of Colour-giving 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. Assay Buffer: 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-Conjugate: Before dilution of the concentrated 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 HRP-conjugate.

3 ml Assay Buffer	30 µl ***Green-Dye***
6 ml Assay Buffer	60 µl ***Green-Dye***
12 ml Assay Buffer	120µl ***Green-Dye***

C. Strepatvidin-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 conjugate dilution. Proceed after addition of ***Red-Dye*** according to the instruction booklet, preparation of Strepatvidin-HRP.

6 ml Assay Buffer	24 µl ***Red-Dye***
12 ml Assay Buffer	48µl ***Red-Dye***

TEST PROTOCOL

a. Prepare your serum and plasmas samples before starting with the test procedure. 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.
Prepare your cell culture supernatants samples before starting with the test procedure. 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.

Sample Matrix	Sample Volume (µl)	Assay Buffer 1x (µl)	HCl 1N (µl)	NaOH 1N (µl)	Dilution
Serum and Plasmas	20	920	30	30	1:500
Cell culture Supernatant	20	180	20	20	1:30

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 sufficient **Microwell Strips coated with Antibody** to mouse TGF-β1 from the aluminium pouch immediately prior to use. Return any wells not required for this assay with desiccant to the pouch. Seal 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 befor aspiration. Take care not to scratch the surface of the microwells.

After the last wash, tap microwell strips on absorbent pad or paper towel to remove excess Wash Buffer. Use the microwell strips immediately after washing or place upside down on a wet absorbent paper for not longer than 15 minutes. Do not allow wells to dry.

d. Add 100 µl of **Assay Buffer**, in duplicate, to all standard wells. Prepare standard dilutions by pipetting 100 µl of **mTGF-β1 Standard**, in duplicate, into well A1 and A2 (see Figure 1 and 2). Mix the contents by repeated aspiration and ejection, and transfer 100 µl to well B1 and B2, respectively. Take care not to scratch the inner surface of the microwells. Mix the contents by repeated aspiration and ejection, and transfer 100 µl to well C1 and C2, respectively. Continue this procedure four times, creating two rows of mTGF-β1 standard dilutions ranging from 2000 to 31.3 pg/ml. Discard 100 µl of the contents from the last microwells (G1, G2) used.

Figure 1. Preparation of mTGF-β1 standard dilutions:

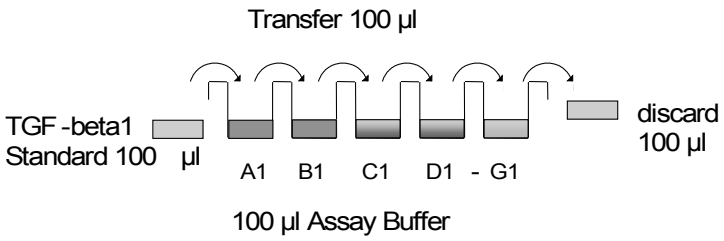


Figure 2. Diagram depicting an example of the arrangement of blanks, standards and samples in the microwell strips:

	Standard Concentrations pg/mL		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	31.3	31.3										
H	Blank	Blank										

e. Add 100 µl of **Assay Buffer**, in duplicate, to the blank wells.

f. For serum and plasmas samples, add 90µl of Assay Buffer to the sample wells. For Cell culture Supernatants samples add 60µl of Assay Buffer to the sample wells.

g. For serum and plasmas samples add 10µl 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.

h. Cover with a **Plate Cover** and incubate at room temperature (18ºC to 25ºC) for 2 hours on **a rotator set at 100 rpm (shaking is absolutely necessary for an optimal test performance)**

i. Prepare **Biotin-Conjugate**. (Refer to preparation of reagents)

j. Remove **Plate Cover** and empty wells. Wash microwell strips 5 times according to point d. of the test protocol. Proceed immediately to the next step.

k. Add 100 µl of diluted **Biotin-Conjugate** to all wells.

l. Cover with a **Plate Cover** and incubate at room temperature (18ºC to 25ºC) for 1 hours on **a rotator set at 100 rpm (shaking is absolutely necessary for an optimal test performance)**

m. Prepare **Strepatvidin-HRP**. (Refer to preparation of reagents)

n. Remove **Plate Cover** and empty wells. Wash microwell strips 5 times according to point d. of the test protocol. Proceed immediately to the next step.

o. Add 100 µl of diluted **Streptavidin-HRP** to all wells, including the blank well.

p. Cover with a **Plate Cover** and incubate at room temperature (18ºC to 25ºC) for 1 hours on **a rotator set at 100 rpm (shaking is absolutely necessary for an optimal test performance)**

q. Remove **Plate Cover** and empty wells. Wash microwell strips 5 times according to point d. of the test protocol. Proceed immediately to the next step.

r. Pipette 100 µl of **TMB Substrate Solution** to all wells, including the blank wells.

m. Incubate the microwell strips at room temperature (18° to 25°C) for about **30 minutes**. Avoid direct exposure to intense light.
The colour development on the plate should be monitored and the substrate reaction stopped (see point o. of this protocol) before positive wells are no longer properly recordable. Determination 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 an OD of 0.9 – 0.95 is reached.

n. Stop the enzyme reaction by quickly pipetting 100 µl of **Stop Solution** into each well, including the blank wells. 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.

o. 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 mTGF-β1 standards.

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.

- Create a standard curve by plotting the mean absorbance for each standard concentration on the ordinate against the mTGF-β1 concentration on the abscissa. Draw a best fit curve through the points of the graph.

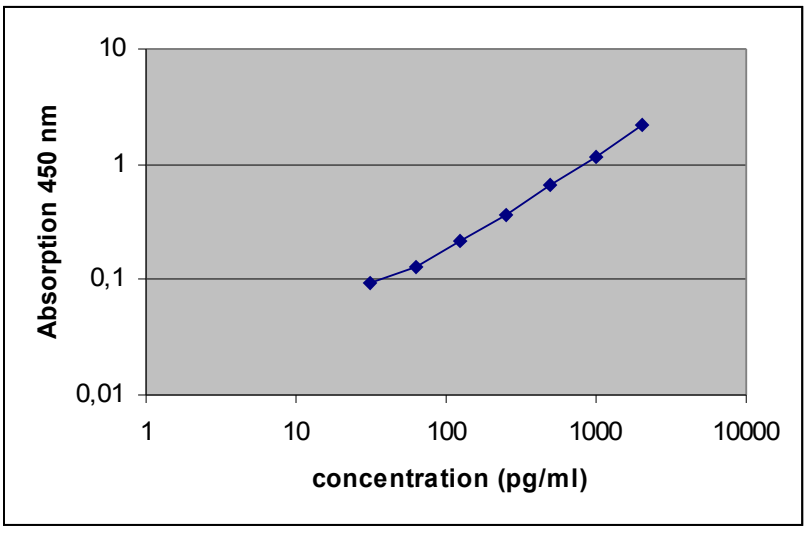
- To determine the concentration of circulating mTGF-β1 for each sample, first find the mean absorbance value on the ordinate and extend a horizontal line to the standard curve. At the point of intersection, extend a vertical line to the abscissa and read the corresponding mTGF-β1 concentration.

- **For serum and plasma samples which have been diluted according to the instructions given in this manual (e.g. 1:500) the concentration read from the standard curve must be multiplied by the dilution factor (e.g. x500). Cell culture supernatant samples have been diluted 1:30, the concentration read from the standard curve must be multiplied by 30.**
  Note: Calculation of samples with an O.D. exceeding the range of the standard curve may result in incorrect low mTGF-β1 levels. Such samples require further dilution with Assay Buffer in order to precisely quantitate the actual mTGF-β1 level.

- It is suggested that each testing facility establishes a control sample of known mTGF-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 3. This curve cannot be used to derive test results. Every laboratory must prepare a standard curve for each group of microwell strips assayed.

Figure 3. Representative standard curve for mTGF-β1 ELISA.
Recombinant soluble mTGF-β1 was diluted in Assay Buffer; symbols represent the mean of three parallel titrations. Do not use this standard curve to derive test results. A standard curve must be run for each group of microwell strips assayed.



Typical data using the mTGF-β1 ELISA

Measuring wavelength: 450 nm
Reference wavelength: 620 nm

Standard	mTGF-β1 Concentration (pg/ml)	O.D. (450 nm)	O.D. Mean	C.V. (%)
1	2000	2.176	2.170	0.3
	2000	2.164		
2	1000	1.150	1.181	2.7
	1000	1.213		
3	500	0.647	0.665	2.7
	500	0.683		
4	250	0.360	0.364	0.9
	250	0.367		
5	125	0.225	0.217	3.6
	125	0.209		
6	62.5	0.132	0.131	0.4
	62.5	0.131		
7	31.3	0.093	0.094	1.4
	31.3	0.096		
Blank	0	0.039	0.039	0.4
	0	0.039		

LIMITATIONS OF THE PROCEDURE

- 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 detergent before use.
- 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 Wash Buffer, fill with Wash Buffer as indicated for each wash cycle and do not allow wells to sit uncovered or dry for extended periods.

PERFORMANCE CHARACTERISTICS

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 9 pg/ml (mean of 6 independent assays).

Reproducibility
a. Intra-assay
Intra-assay variability was determined by 6 replicates of 8 serum samples. The average coefficient of variation was 5.8%.

b. Inter-assay
Inter-assay variability was determined by 6 replicates of 8 serum samples. The average coefficient of variation was 10.8%.

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.

Spike Recovery

For recovery data see table below

Sample Matrix	Spike high (%)	Spike medium (%)	Spike low (%)
Serum	92	103	102
Plasmas (EDTA)	93	96	105
Plasmas (citrate)	96	94	105
Cell culture Supernatant	124	141	131

Dilution Linearity

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

Sample Matrix	Range (%) Recovery of EXp.Val	Mean(%)
Serum	78-113	93
Plasma (EDTA)	86-126	105
Plasma (citrate)	105-115	110
Cell culture supernatant	90-115	106

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Sample Stability

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

Storage Stability : Aliquots of Serum samples (spiked or unspiked) were stored at -20°C, 2-8°C and room temperature (RT) and the mTGFβ1 determined after 24h. There was no significant loss of immunoreactivity detected during storage under above conditions.

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.

REAGENT PREPARATION SUMMARY

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

B. Assay Buffer

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

C. Biotin-Conjugate

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

D. Strepatvidin-HRP

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

D. Standard Dilute Standard by addition of **Distilled Water** according to vial label.

TEST PROTOCOL SUMMARY

- Pretreat Samples (1h)
- Wash Microwell Strips twice with **Wash Buffer**
- Add 100 µl **Assay Buffer**, in duplicate, to all standard wells
- Pipette 100 µl **mTGF-β1 Standard** into the first standard wells and create standard dilutions ranging from 2000 to 31.3 pg/ml by transferring 100 µl from well to well; discard 100µl from the last wells.
- Add 100 µl **Assay Buffer**, in duplicate, to the blank wells
- Add 10 µl **pretreated serum and plasma samples** to designated wells containing 90 µl of Assay Buffer and 40µl **pretreated cell culture supernatant samples** to designated wells containing 60 µl of Assay Buffer.
- Cover microwell strips and incubate 2 hours at room temperature (18° to 25°C) **on a rotator**
- Prepare Biotin Conjugate
- Empty and wash microwell strips 5 times with **Wash Buffer**
- Add 100µl Biotin- Conjugate to all wells
- Cover microwell strips and incubate 1 hours at room temperature (18° to 25°C) **on a rotator**
- Prepare Streptavidin-HRP
- Empty and wash microwell strips 5 times with **Wash Buffer**
- Add 100µl Streptavidin-HRP to all wells
- Cover microwell strips and incubate 1 hours at room temperature (18° to 25°C) **on a rotator**
- Empty and wash microwell strips 5 times with **Wash Buffer**
- Add 100 µl of **TMB Substrate Solution** to all wells including blank wells
- Incubate the microwell strips for about 30 minutes at room temperature (18° to 25°C)
- Add 100 µl **Stop Solution** to all wells including blank wells
- Blank microwell reader and measure colour intensity at 450 nm

Note: For samples which have been diluted according to the instructions given in this manual 1:500 serums and plasmas or 1:30 cell culture supernatant the concentration read from the standard curve must be multiplied by the dilution factor (x500, x30 respectively).