biosensis® Mouse Transforming growth factor beta-1/TGF-beta-1 (TGFβ1) ELISA

Kit Protocol

Catalog No: BEK-2095-2P

For quantitative detection of mouse TGFβ1 in cell culture supernatants, serum, EDTA treated plasma, and urine samples only when used as directed

TABLE OF CONTENTS

I. Materials Provided

Kit Components	Unit Size/Volume
Lyophilized recombinant mouse TGFβ1 standard	10ng/tube x 4
96 wells plate pre-coated with anti-mouse TGFβ1 antibody	2 plates
Sample diluent buffer	30ml x 2
Biotinylated anti- mouse TGFβ1 antibody (dilution 1:100)	100µl x 2
Antibody diluent buffer	12ml x 2
Avidin-Biotin-Peroxidase Complex (ABC) (dilution 1:100)	100µl x 2
ABC diluent buffer	12ml x 2
TMB colour developing agent	10ml x 2
TMB stop solution	10ml x 2
Wash Buffer Concentrate (25x)	20ml x 2
Plate Sealers	1 ea x 8

WARNING: DO NOT MIX REAGENTS SUCH AS ANTIBODY BUFFERS ETC BETWEEN DIFFERENT ASSAYS IF USING MULTIPLE KITS. USE ONLY THE BUFFERS, STANDARDS & SOLUTIONS INCLUDED FOR YOUR SPECIFIC LOT. MIXING SOLUTIONS FROM DIFFERENT TARGET KITS CAN LEAD TO POOR KIT PERFORMANCE

II. Equipment Required but Not Supplied

1. Distilled Water, 1000 mL +, graduated cylinders for measuring
2. Automated plate washer is desirable
3. Multi-channel or repeating pipette.
4. Clean tubes and Eppendorf tubes.
5. Plate shaker (300-500 rpm)
6. Mechanical Vortex.
7. Microplate reader with 450nm filter.

III. Technical Hints

1. Spin all kit components to get liquid to the bottom of tubes before use.

2. Duplicate well assays are recommended for both standard and sample testing.

3. In order to avoid marginal effects of plate incubation due to temperature differences, it is suggested that the ABC and TMB solutions be brought to room temperature (or 37° C if using option protocol) 30 min before use.

4. Recommended method for manual plate washing.

- Emptying of wells on the plate:
  Place the plate on the palm of the hand in a position that enables easy flicking movement using the wrist. Holding the plate over a sink, quickly invert the plate, whilst accelerating the arm downward toward the sink. Abruptly stop the downward acceleration to force the liquid from the wells into the sink. When done correctly the technique should prevent liquid from getting on to the fingers or on the outside of the strip wells or plate holder. *Note: Retain the upside down position of the plate to avoid any back flow into the wells. DO NOT LET THE PLATE DRY OUT.*

- Blotting the plate:
  Immediately blot the inverted plate by lightly tapping the plate 3-4 times on the blotting paper.

- Washing: Forcefully pipette Wash Buffer into each well with a multi-channel pipette. Empty the wells of wash buffer using the technique described above. Repeat washing and flicking procedures thrice. Wash the plate with 0.01M PBS, and each time let the washing buffer stay in the wells for 1-2 min before rinsing with more wash buffer as directed above. *Note: **Avoid** touching the inside surface of the wells with the pipette tips.*
  Do not let the wells dry out at any time or enzymatic activity will be lost.

5. Preparation of Wash buffer:

- Prepare 500 ml of Working Wash Buffer by diluting the supplied 20 ml of Wash Buffer (25 x) with 480 ml of deionized or distilled water. If crystals have formed in the concentrate, warm to room temperature and mix it gently until crystals have completely dissolved.

Note: if blank values are over 0.2, adding 0.05% Tween-20 PBS wash buffers will usually help lower them and overall assay backgrounds and can help improve signal/noise ratios.

IV. Storage of Kit Components

This kit may be stored for up to 6 months at 2°C to 8°C from the date of shipment. Standards should be stored at -20°C or -80°C (recommended at –80°C) after reconstitution. Coated strips or reagents may be store for up to 1 month at 2°C to 8°C. Return unused wells to the pack and reseal pack. *Note: the kit can be used within 8 months if the whole kit is stored at -20°C. Avoid repeated freeze-thaw cycles.*

Special Notes: Discard the Tgfb1 stock solution after 12 hours at 4°C. May be stored at -20°C for 48 hours. Avoid freeze thaw cycles as TGFb1 is fragile.

V. Sample Preparation and Dilution

- **Cell culture supernatant:** Remove particulates by centrifugation (10,000 x g 5 min), analyze immediately or aliquot and store at -20°C-80°C. Divide samples into aliquots to prevent multiple freeze thaws; use appropriate protease inhibitor cocktails for best results. **Note**: Animal serum used in the preparation of cell culture media may contain high levels of latent TGF-beta 1. For best results, do not use animal serum for growth of cell cultures when assaying for TGF-beta-1 production. If animal serum is used as a supplement in the media, precautions should be taken to prepare the appropriate control and run the control in the immunoassay to determine the baseline concentration of TGF-beta-1.

- **Serum:** Allow the serum to clot in a serum separator tube (about 30 minutes-4 hours) at room temperature. Centrifuge at approximately 1000 X g for 10 min. Analyze the serum immediately or aliquot and store frozen at -20°C-80°C.
- **Plasma:** Collect plasma using EDTA as an anticoagulant. Centrifuge for 15 min. at 1500 x g within 30 min. of collection. Assay immediately for divide into aliquots and store at -20°C-80°C.
- **Urine**: Aseptically collect the first urine of the day, and micturate directly into a sterile container. Remove particular impurities by centrifugation, assay immediately or aliquot and store samples at -20°C. *Note: treatment of urine with Thiomersal or other proteinase inhibitors can increase signal yields in ELISA assays for some targets, please see: (Hoyer, J. et al. (1999). Effects of microgravity on urinary osteopontin. J. Am. Soc. Nephrol. 10: S389-S393.)*

- **Activating Reagent: In samples, TGF beta-1 is mostly contained as an inactive protein-bound form**. It is necessary to activate via acidification before using the samples in the ELISA assay. Free natural TGF beta is highly liable. It is not necessary to activate the recombinant TGF-beta-1 standard included in this ELISA, as it is already free of inactivating binding proteins. Still, if desired, one can treat it to ensure similar reactivity and function as a control.

Needed solutions: Solution A: 1N HCl, pH < 1.0: add 8.33ml of 12N HCl into 91.67ml of H2O. **Solution B:** 1.2N NaOH/0.5M HEPES, pH 14: add 12ml of 10N NaOH and 11.9g HEPES into 75ml of H2O, add H2O to adjust the volume to 100ml.

Activation/Acidification the samples:

Cell culture supernate, urine: (1:5 treatment ratio): Add activating reagent proportionally, i.e. add 20 microliters of Solution A into 100 microliters of undiluted sample, 10 min later, add 20 microliters of Solution B to the tube to neutralize the acidification step. If precipitate forms, samples can be centrifuged at 16,000 x g for 5 minutes at 4 degrees centigrade to pellet any material. Use only the clear supernatants for the ELISA. The final pH of the sample solution should be ~7.0 to 7.6 for use in the ELISA. Use pH paper or microprobe to measure final pH.

Serum, plasma (EDTA): **(1:2 treatment ratio)** Add activating reagent proportionally, i.e. add 20 microliters of Solution A into 40 microliters of undiluted sample, 10 min later, add 20

microliters of Solution B to the tube to neutralize the acidification step. If precipitate forms, samples can be centrifuged at 16,000 x g for 5 minutes at 4 degrees centigrade to pellet any material. Use only the clear supernatants for the ELISA. The final pH of the sample solution should be ~7.0 to 7.6 for use in the ELISA. Use pH paper or microprobe to measure final pH.

Note: Sample activation will dilute experimental samples partly after adding activating reagent, so please pay attention to this when calculating target protein concentration for the most accurate results.

Also, the user needs to estimate the concentration of the target protein in the sample and select a proper dilution factor so that the diluted target protein concentration falls near the middle of the linear regime in the standard curve. Also with certain growth factors and target proteins, binding proteins and location can influence over all signal. It is recommended that researchers confirm by literature an effective lysis, cell membrane preparations and/or extraction buffer formulations and treatments that are suitable for the precise target being assayed.

The following table can be used as a guideline for sample dilution. Dilute the sample using the provided diluent buffer. Mix the sample thoroughly with diluent buffer.

Target protein concentration	**Concentration range**	**Working dilution**	**Amount of sample diluent buffer to be added**
High target protein concentration	**10-100 ng/ml**	1:100	Add 1 µl sample into 99 µl of sample diluent buffer
Medium target protein concentration	**1-10 ng/ml**	1:10	Add 10 µl sample into 90 µl of sample diluent buffer
Low target protein concentration	**15.6-1000 pg/ml**	1:2	Add 50 µl sample to 50 µl of sample diluent buffer
Very Low target protein concentration	**≤15.6 pg/ml**	No dilution necessary	(working dilution is 1:2)

Assay Precision:

	Intra-Assay Precision			Inter-Assay Precision		
Sample	1	2	3	4	5	6
n	16	16	16	24	24	24
Mean (pg/mL	36	169	499	33	183	484
Std. Dev.	1.47	11.66	31.43	1.55	12.99	34.36
CV(%)	4.1	6.9	7.1	4.7	7.1	7.1

Range 15.6 pg/mL to 1000 pg/mL; Sensitivity: < 2 pg/mL. Cross reactivity: < 1% with TGF-beta 2,TGF-beta 3, TGF-beta 5. Not expected to react with Rat TGF-beta 1 but untested.

VI. Reagent Preparation

1. Preparation of Standards

- Reconstitution of standard with the sample diluent provided.

  (a) 10,000 pg/ml (10 ng/ml) of mouse TGFβ1 standard solution: Add 1ml of sample diluent buffer into ***one*** tube of standard, mix thoroughly and keep the tube at room temperature for 10 min. Standard is recombinant mouse TGFB1 chain, amino acids 279-390 expressed in CHO expression system and purified.

  (b) 1000pg/ml of human TGFβ1 standard solution: Add 0.1ml of the above 10ng/ml TGF-beta-1 standard solution into 0.9ml sample diluent buffer and mix thoroughly.

  (b) 500 pg/ml to 15.6 pg/ml of human TGFβ1 standard solutions: Label 6 Eppendorf tubes with 500 pg/ml, 250 pg/ml, 125 pg/ml, 62.5 pg/ml, 31.2 pg/ml, 15.6 pg/ml respectively. Aliquot 0.3 ml of sample diluent buffer into each tube. Transfer 0.3ml from 1st tube into 2nd and mix. Continue performing serial dilutions.

- *Note: Standard solution should be prepared no more than 2 hours prior to the experiment. The working standard solution may be stored at 4°C for up to 12 hours, or at -20°C for up to 48 hours. Avoid repeated freeze-thaw cycles.*

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2. Preparation of Biotinylated antibody working solution

- The total volume should be: 0.1ml/well x (the number of wells). (Allowing 0.1-0.2 ml more than total volume).
- Biotinylated antibody (Reagent B) should be diluted in 1:99 with the antibody diluent buffer and mixed thoroughly.
- *Note: the solution should be prepared no more than two hours prior to the experiment.*

3. Avidin-Biotin-Peroxidase Complex (ABC)

- The total volume should be: 0.1ml/well x (the number of wells). (Allowing 0.1-0.2 ml more than total volume).
- ABC should be diluted in 1:99 with the ABC diluent buffer and mixed thoroughly.
- *Note: the solution should be prepared no more than one hour prior to the experiment.*

VII. Assay Procedure

We recommend that standard solution dilutions and each sample are plated in duplicate. Read entire protocol before beginning; if using optional method the ABC working solution and TMB color developing agent must be kept warm at 37°C for 30 min before use.

1. Aliquot out the standards into each well of the pre-coated plate. Add 0.1ml of the sample diluent buffer into the control well (Zero well). Add 0.1ml of each properly diluted sample into each empty well. We recommend at least duplicate standards and samples. *Note: See "VII. Sample Preparation and Dilution Guideline" above for details.*

2. Seal the plate with parafilm and incubate the plate at 4°C overnight. *Note: we recommend users leave the plate on a shaker if possible.* (Optional method: 37°C, 90 minutes but it is slightly less sensitive)

3. Remove the cover, discard the plate contents and blot the plate onto paper towels or other absorbent material. **DO NOT** let the wells completely dry at any time. Wash plate 3-5 times using wash buffer as described in the technical hints. *Note: See "V. Technical Hints - Recommended method for manual plate washing" above for details.*

4. Add 0.1ml of biotinylated antibody working solution into each well. Cover and incubate the plate at room temperature on a plate shaker for 2-3 hours. (Optional 37°C, 60 minutes with shaking)Wash 3X as described in the technical hints.

5. Add 0.1ml of prepared ABC working solution (*Note: See "VIII. Reagent Preparation"* above for details) into each well. Cover and incubate the plate at room temperature on a plate shaker for 1 hour. (Optional 37°C, 30 minutes with shaking). Wash 5X as described in technical hints allowing the wash buffer to remain in the wells for 1-2 minutes.

6. Warm TMB to room temperature (37° if using optional protocol). Add 90 ul of prepared TMB color developing agent into each well and incubate **in the dark** for 15-35 minutes at RT (or 15-20 minutes at 37°C). The optimal incubation time must be empirically determined. A guideline to look for is blue shading for the top four standard wells, while the remaining standards remain clear. Stopping the reaction too quickly can lead to low standard curve vales. Note: depending upon the sample dilution the experimental samples may show color much faster, if this is the case repeating the assay at a lower sample dilution is recommended if sample O.Ds are out of range.

7. Stop the reaction by adding 0.1ml of prepared TMB stop solution into each well. The color will change into yellow.

8. Immediately read the plate at 450nm with a microplate reader. *Note: Color will fade over time; hence, we recommend plate to be read within 30 min after adding the stop solution*.

CAUTION: Bubbles in the wells will cause inaccurate readings. Ensure that all bubbles are removed prior to taking the absorbance reading.

Additional use Notes:

1. Solutions: To avoid cross-contamination, change pipette tips between additions of each standard, between sample additions, and between reagent additions. Also, use separate reservoirs for each reagent.

2. Applying Solutions: All solutions should be added to the bottom of the ELISA plate well. Avoid touching the inside wall of the well. Avoid foaming when possible.

3. Assay Timing: The interval between adding samples to the first and last wells should be minimized. Delays will increase the incubation time differential between wells, significantly affecting the experimental accuracy and repeatability. For each step in the procedure, the total dispensing time for the addition of reagents or samples should not exceed 10 minutes.

4. Incubation: To prevent evaporation and ensure accurate results, proper adhesion of plate sealers during incubation is necessary. Do not allow wells to sit uncovered for extended periods of time between incubation steps. Do not let wells dry out at any time during the assay. Strictly observe the recommended incubation times and temperatures.

5. Washing: Proper washing procedure is critical. Insufficient washing will result in poor precision and falsely elevated absorbance readings. Residual liquid in the reaction wells should be patted dry against absorbent paper during the washing process. Do not put absorbent paper directly into the reaction wells.

6. Controlling Substrate Reaction Time: After adding the TMB Substrate, periodically monitor the color development. Stop color development before the color becomes too deep by adding Stop Solution. The excessively strong color will result in inaccurate absorbance readings.

7. Reading: The microplate reader should be preheated and programmed before use. Before taking O.D. readings, remove any residual liquid or fingerprints from the underside of the plate and confirm that there are no bubbles in the wells.

8. Reaction Time Control: Control reaction time should be strictly followed as outlined.

9. Stop Solution: The Stop Solution contains an acid. Therefore proper precautions should be taken during its use, such as protection of the eyes, hands, face, and clothing.

10. To minimize the external influence on assay performance, operational procedures and lab conditions (such as room temperature, humidity, and incubator temperature) should be strictly controlled. It is also strongly suggested that the same operator performs the whole assay from the beginning to the end.

VIII. Calculation of Results

(a) Manual Plate Reading:

(The relative $O.D._{450}$) = (the $O.D._{450}$ of each well) – (the $O.D._{450}$ of Zero well).

The standard curve can be plotted as the relative $O.D._{450}$ of each standard solution (Y) vs. the respective concentration of the standard solution (X). Known concentrations of the target protein are plotted on the X-axis and the corresponding $O.D._{450}$ on the Y-axis. The standard curve should result in a graph that shows a direct relationship between target protein concentrations and the corresponding $O.D._{450}$. The greater the concentration of target protein in the sample, the higher the $O.D._{450}$.

Note: if the samples measured were diluted, multiply the dilution factor to the concentrations from interpolation to obtain the concentration before dilution.

Determine concentration of target protein in unknown sample:

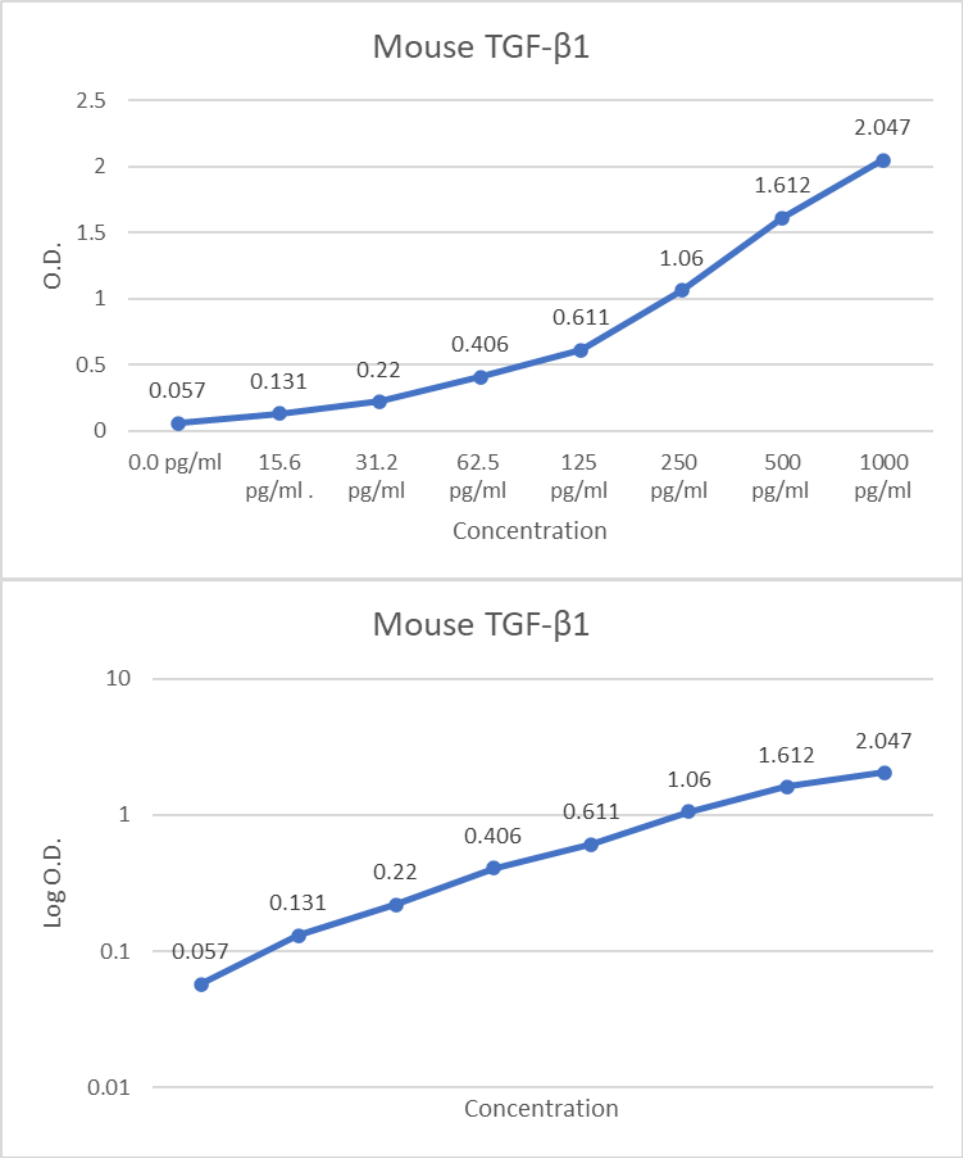
The target protein concentration of the samples can be interpolated from the standard curve. Draw a horizontal line to intersect with the standard curve. A vertical line dropped from this point intersects the X-axis at the concentration of antibody in the unknown sample.

(b) PC Interface Plate Reading

Enter the data into computer program curve fitting software. Use appropriate software to reduce the data and generate a four parameter logistic (4-PL) curve-fit; avoid using linear regression analysis. Perform a 4-PL regression analysis to calculate the concentration of target analyte in the samples. Multiply the result by the sample dilution factor

IX: Typical Standard Curve (for reference only, not to be used for actual data)

Concentration pg/ml	0.0 pg/ml	15.6 pg/ml .	31.2 pg/ml	62.5 pg/ml	125 pg/ml	250 pg/ml	500 pg/ml	1000 pg/ml
O.D.	0.057	0.131	0.220	0.406	0.611	1.060	1.612	2.047



(TMB reaction incubate at 37°C for 25-30 min)