biosensis® Mouse Thrombopoietin/TPO/Megakaryocyte growth and development factor/MGDF ELISA

Kit Protocol

Catalog No: BEK-2096-2P

For quantitative detection of mouse thrombopoietin in cell culture supernatants, cell and tissue homogenates, serum and heparin, or EDTA treated plasma samples only when used as directed.

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I. Materials Provided

Kit Components	Unit Size/Volume
Lyophilized recombinant mouse TPO standard	10ng/tube x 4
96 wells plate pre-coated with anti-mTPO antibody	2 x plates
Sample diluent buffer	30ml x 2
Biotinylated anti-mTPO antibody (dilution 1:100)	130µl x 2
Antibody diluent buffer	12ml x 2
Avidin-Biotin-Peroxidase Complex (ABC) (dilution 1:100)	130µl x 2
ABC diluent buffer	12ml x 2
TMB colour developing agent	10ml x 2
TMB stop solution	10ml x 2

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. Wash buffer (0.01M PBS or TBS). *See technical hints*
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 blotting paper.

- Washing: Forcefully pipette Wash Buffer into each well with a multi-channel pipette. Empty the wells of wash buffer using technique described above. Repeat washing and flicking procedures thrice. Wash plate 0.01M TBS or 0.01M PBS, and each time let 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: (not provided in the kit)

Preparation of 0.01M **TBS:** Add 1.2g Tris, 8.5g Nacl; 450µl of purified acetic acid or 700µl of concentrated hydrochloric acid to 1000ml H_2O and adjust pH to 7.2-7.6. Finally, adjust the total volume to 1L.

Preparation of 0.01 M **PBS:** Add 8.5g sodium chloride, 1.4g Na_2HPO_4 and 0.2g NaH_2PO_4 to 1000ml distilled water and adjust pH to 7.2-7.6. Finally, adjust the total volume to 1L.

Note: PBS buffers typically work more reliably than TBS buffers in most cases and are recommended.
Note: if blank values are over 0.2 the addition of 0.05% Tween-20 to either TBS or PBS wash buffers will usually help lower them as well as 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.*

V. Sample Preparation and Dilution

- **Serum:** Allow the serum to clot in a serum separator tube (about 4 hours) at room temperature. Centrifuge at approximately 1000 X g for 15 min. Analyze the serum immediately or aliquot and store samples at -20°C.
- **Plasma:** Collect plasma using **heparin or EDTA** only as an anticoagulant. Centrifuge for 15 min at 2-8℃ at 1500 x g within 30 min of collection. Analyze immediately or aliquot and store samples at -20°C-80°C.
- **Cell culture supernatant:** Remove particulates by centrifugation (10,000 x g 5 min), analyze immediately or aliquot and store at -20°C-80°C.
- **Cell lysates**: Lyse with RIPA or another non-denaturing lysis buffer. The tube liquid needs to be clear before using in the ELISA. Spin at 10,000xG for 5-10 minutes a 4°C; aliquot cleared supernatants or use immediately following dilutions as recommended. RIPA typically requires a minimum dilution of 1:10. Use of proteinase inhibitor cocktail is recommended;check final pH is neutral to 7.5. Analyze immediately or aliquot and store frozen at -20°C-80°C.
- **Tissue**: Wash freshly dissected tissue in ice-cold PBS several times. Homogenize tissue by sonication or maceration or both. Recommend 10:1 lysis buffer to tissue weight ratio, i.e. 10 mL: 1g tissue). Supernatant needs to clear and free of particles for use in ELISA. Analyze the sample immediately or aliquot and store frozen at -20°C-80°C. Use of proteinase inhibitor cocktail is recommended; check final sample pH is 7.0-7.5 for best results. RIPA receipe: 150 mM sodium chloride; 1.0% NP-40 or Triton X-100; 0.5% sodium deoxycholate; 0.1% SDS (optional); 50 mM Tris, pH 8.0)

Note: 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	**20-200 ng/ml**	1:100	Add 1 µl sample into 99 µl sample diluent buffer
Medium target protein concentration	**2-20 ng/ml**	1:10	Add 10 µl sample into 90 µl sample diluent buffer
Low target protein concentration	**31.2-2000 pg/ml**	1:2	Add 50 µl sample to 50 µl sample diluent buffer
Very Low target protein concentration	**≤31.2 pg/ml**	No dilution necessary	(working dilution is 1:2)

	Intra-Assay Precision			**Inter-Assay Precision**		
Sample	1	2	3	1	2	3
N	16	16	16	24	24	24
Mean (pg/ml)	296	611	1265	321	746	1464
Stan. Dev.	11.84	33	82.23	16.7	47	105.4
CV(%)	4	5.4	6.5	5.2	6.3	7.2

Range 31.2-2,000 pg/ml; Sensitivity < 5 pg/mL Specificity: mouse TPO, no detectable cross-reactivity with other relevant proteins.

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 TPO 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 TPO produced in a mammalian cell expression system and purified, aa.22-356 of mouse TPO.

  (b) 2000pg/ml of Mouse Thrombopoietin/TPO standard solution: Add 0.2ml of the above Thrombopoietin/TPO standard solution into 0.8 ml sample diluent buffer and mix thoroughly.

  (c) 1000 pg/ml to 31.25 pg/ml of mouse TPO standard solutions: Label 6 Eppendorf tubes with 1000 pg/ml, 500 pg/ml, 250 pg/ml, 125 pg/ml, 62.5 pg/ml, 31.25 pg/ml respectively. Aliquot 0.3 ml of sample diluent buffer into each tube. THEN Add 0.3 ml of the above 2000 pg/ml standard solution (item a) into the 1st tube and mix. 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.*

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 90µl of prepared TMB color developing agent into each well and incubate plate at room temperature for 5-20 min in the dark (shades of blue can be seen in the wells with the four most concentrated standard solutions; the other wells show no obvious color).
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.

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:

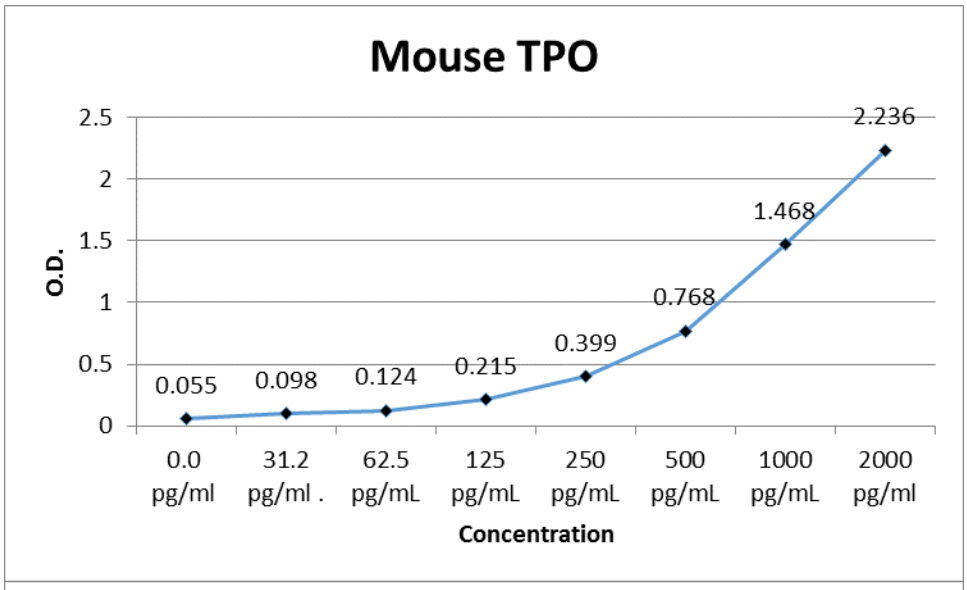
The target protein concentration of the samples can be interpolated from the standard curve. Draw a horizontal line to intersect with the standard curve. A vertical line dropped from this point intersects the X-axis at the concentration of antibody in the unknown sample.

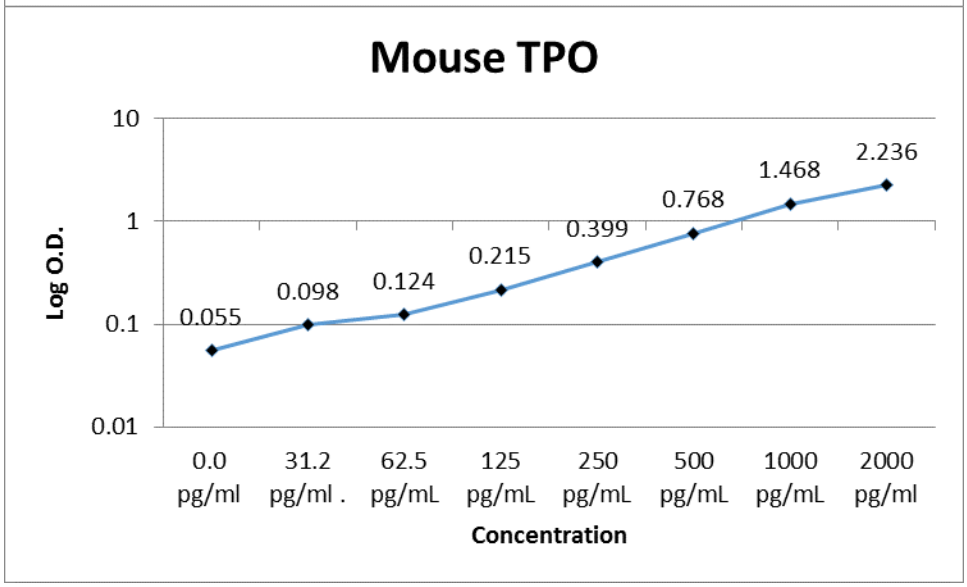
(b) PC Interface Plate Reading

Enter the data into computer program curve fitting software. Existing spreadsheet software can perform a good fit with a linear regression analysis.

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

Concentration pg/ml	0.0 pg/ml	31.2 pg/ml .	62.5 pg/mL	125 pg/mL	250 pg/mL	500 pg/mL	1000 pg/mL	2000 pg/ml
O.D.	0.055	0.098	0.124	0.215	0.399	0.768	1.468	2.236





TMB reaction 37°C, 25-30 minutes.