CELL APPLICATIONS, INC.

Mouse TP0 ELISA Kit

Cat. No. CL0517
96-wells

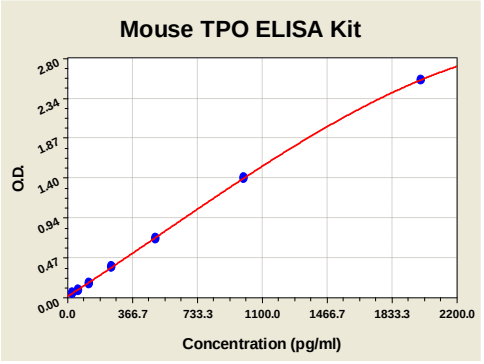
Target Protein Species:	Mouse
Range	31.2pg/ml – 2000pg/ml
Specificity	No detectable cross-reactivity with any other cytokine

KIT COMPONENTS

Component	Amount
96-well plate precoated with anti-mouse TP0 antibody	1 Plate
Protein Standard: Lyophilized recombinant mouse TP0	2 tubes, 10 ng/tube
Sample Diluent Buffer	30 ml
Biotinylated Antibody (Anti-mouse TP0)	130 µl (100x)
Antibody Diluent Buffer	12ml
Avidin-Biotin-Peroxidase Complex (ABC) Solution	130 µl (100x)
ABC Diluent Buffer	12 ml
Tetramethyllbenzidine (TMB) Color Developing Agent	10 ml
TMB Stop Solution	10 ml

Storage

Store at 4℃. Cell Applications, Inc. recommends using the kit within 6 months of order.



X	pg/ml	0.0	31.2	62.5	125	250	500	1000	2000
Y	O.D.450	0.022	0.060	0.104	0.181	0.376	0.703	1.408	2.551

Figure 1: TP0 Standard Curve. Using the mouse TP0 ELISA Kit, O.D. data was graphed against TP0 protein concentration. The TMB reaction was incubated at 37° C for 20 min.

BACKGROUND

Thrombopoietin (TPO) is a novel hematopoietic growth factor and a ligand for the c-mpl proto-oncogene that was originally identified as the cellular homologue of the oncogene v-mpl proto-oncogene. The c-mpl proto-oncogene is expressed in hematopoietic tissues, particularly in CD34+ hematopoietic progenitor cells, megakaryocytes, and platelets, whereas its ligand TPO is detected primarily in the liver, kidney, and smooth muscle, with lesser amounts present in the spleen and bone marrow.[1] It has been shown that daily infusion of TPO into mice or nonhuman primates induces a marked increase in the counts of platelets, megakaryocytes, and megakaryocytic progenitor cells. Furthermore, c-Mpl- or TPO-deficient mice generated by gene targeting have been demonstrated to exhibit a striking decrease in the number of platelets and megakaryocytic progenitor cells. Interestingly, in the absence of Tpo or c-mpl, the residual megakaryocytes and platelets are morphologically and functionally normal.Therefore, Tpo/mpl is important for controlling megakaryocyte numbers by promoting their survival and/or proliferation as well as differentiation, but is not essential for megakaryocyte cytoplasmic maturation or platelet formation.[2]

c-Mpl belongs to the type I cytokine receptor family. Ligand binding induces activation of the JAK2 that is associated with the receptor through Box1 and Box2. Activated JAK2 phosphorylates tyrosine residues on cytokine receptors, thereby providing docking sites for several Src homology 2 (SH2) domain–containing downstream signaling proteins. Mpl activates many signaling pathways in hematopoietic cell lines and primary megakaryocytes and platelets, including STAT3 and 5, Shc–Ras–mitogen-activated protein kinase (MAPK), and SHP2–Gab–phosphoinositide-3 kinase–Akt pathways. On the other hand, Lnk, an adptor protein, negatively regulates thrombopoietin (TPO) signaling in megakaryocytes and erythropoietin signaling in erythroblasts. Thus, it is suggested that a balance in positive and negative signals downstream from the TPO signal plays a role in the regulation of the probability of self-renewal in hematopoietic stem cells. These signaling molecules have important roles in hematopoiesis, and dysregulation of these signaling pathways is implicated in leukemogenesis.[3]

ELISA OVERVIEW

Cell Applications ELISA Kits are based on standard sandwich enzyme-linked immunosorbent assay technology. Freshly prepared standards, samples, and solutions are recommended for best results.

1. Prepare test samples.
2. Prepare a protein standard of the target protein.
3. Add test samples and standards to the pre-coated 96-well plate. Do not wash.
4. Add biotinylated detection antibodies. Wash.
5. Add Avidin-Biotin-Peroxidase Complex (ABC) Solution. Wash.
6. Add Tetramethyllbenzidine (TMB) Color Developing Agent, containing HRP substrate.
7. Add TMB Stop Solution
8. Subject the plate to analysis.

NOTES:

- Before using the kit, quick spin tubes to bring down all solution to the bottom of tube.
- Duplicate assay wells are recommended for both standard and sample testing.
- Do not let the 96-well plate dry, this will lead to inactivation of plate components.
- When diluting samples and reagents, ensure that they are mixed completely and evenly.
- Pre-warm diluted ABC and TMB solutions at 37℃ for 30 min before use to avoid variable temperature effects.
- For washes, use TBS or PBS. Do not touch well walls.
- A protein standard is included in the kit. A protein standard detection curve should be generated with each experiment, no more than 2 hours prior to the experiment.
- The user will determine sample dilution fold by estimation of target protein amount in samples.

Reference

1. Kaushansky, K. & Drachman, J. G.: Oncogen 21:3359-67, 2002
2. Matsumura, I. et al: Mol. Cell. Biol.17:2933-43, 1997
3. Seita, J. et al: Proc. Natl. Acad. Sci. USA 104:2349-54, 2007

ELISA PROTOCOL

Preparation of Test Samples

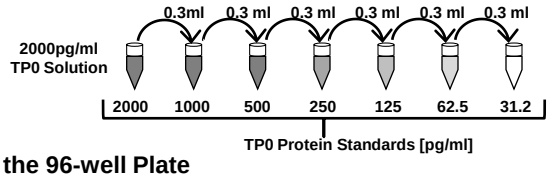
1. Process Test Samples in the following manner:
   - **Cell culture supernate, tissue lysate or body fluids**: Remove particulates by centrifugation.
   - **Serum**: Allow the serum to clot in a serum separator tube (about 30 min) at room temperature. Centrifuge at approximately 2000 x g for 20 min. Analyze the serum immediately or aliquot and store frozen at -20° C.
2. Estimate the concentration of the target protein in the sample and select a proper dilution factor such that the diluted target protein concentration falls within the 31.2-2000 pg/ml standard curve range. Depending on the sample, several trial dilutions may be necessary. Dilute the sample using the provided diluent buffer, mixing well. Suggested working dilutions of samples are as follows:

Target Protein Concentration Range	Sample Working Dilution	Sample Vol.	Diluent Buffer Vol.
20-200ng/ml	1:100	1 µl	99 µl
2-20 ng/ml	1:10	10 µl	90 µl
31.2-2000 pg/ml	1:2	50 µl	50 µl
≤31.2 pg/ml	n/a	100µl	n/a

3. If samples will be assayed within 24 hours, store at 2-8° C. For long-term storage, aliquot and freeze samples at -20° C. Avoid repeated freeze-thaw cycles.

Preparation of Standard Solutions (31.2-2000 pg/ml)

4. Reconstitute the Lypophilized Recombinant Protein to make a 10.000 pg/ml mouse TP0 solution. Add 1 ml Sample Diluent Buffer to a tube of lypophilized protein, keep the tube at room temperature for 10 min. Mix thoroughly.
5. Add 0.2 ml of the mixed 10,000 pg/ml TP0 solution to the eppendorf tube containing 0.8 ml diluent buffer and mix to make a 2000 pg/ml TP0 solution
6. Label 6 eppendorf tubes with the TP0 protein concentrations to be prepared by serial dilution: 1000pg/ml, 500pg/ml, 250pg/ml, 125pg/ml, 62.5pg/ml, 31.2pg/ml.
7. Aliquot 0.3 ml of the Sample Diluent Buffer to the labeled tubes.
8. Serially dilute the protein standards into their respectively labeled tubes. Transfer 0.3 ml from the 2000pg/ml TP0 Solution to the 1000pg/ml eppendorf tube and mix thoroughly. Transfer 0.3 ml of the 1000 pg/ml solution to the 500pg/ml tube and mix thoroughly, Transfer 0.3 ml of the 500pg/ml solution to the 250pg/ml tube and mix, and so on to make the 125, 62.5 and 31.2 pg/ml solutions.
9. Store at 4° C until use.



Loading the 96-well Plate

10. Aliquot 0.1 ml of the sample diluent buffer into a control well to serve as the Blank. This will yield the O.D.450(Blank) reading.
11. Aliquot 0.1 ml of the standard solutions of the **Preparation of Standard Solutions** (31.2-2000pg/ml) into empty wells of the precoated 96-well plate. Duplicate measurements of standards are recommended.
12. Aliquot 0.1 ml of each properly diluted test sample to empty wells prepared in **Step 2**. Duplicate measurements of each test sample are recommended.
13. Cover the 96-well plate and incubate at 37° C for 90 min.
14. During the **Step 13** incubation period, prepare a stock of Biotinylated 1:100 Antibody Working Solution. Count the number of reactions and multiply by 0.1 ml/well for the Working Solution total volume (preparation of 1-2 reactions in excess of the number of wells is recommended). Dilute the Biotinylated Antibody to 1:100 in Antibody Diluent Buffer and mix thoroughly. Use the working solution within 2 hours.
15. Upon completion of the 90 min incubation of **Step 13**, remove the cover of the 96 well plate and discard plate well contents. Blot the plate onto paper towels or other absorbent material. DO NOT let the wells completely dry at any time.
16. Add 0.1 ml of the Biotinylated 1:100 Antibody Working Solution (prepared in **Step 14**) to each well and incubate the plate at 37° C for 60 min.
17. During the incubation period of **Step 16**, prepare a stock of ABC Working Solution. Count the number of reactions and multiply by 0.1 ml/well for the Working Solution total volume (preparation of 1-2 reactions in excess of the number of wells is recommended). Dilute the ABC Stock Solution to 1:100 in ABC Diluent Buffer and mix thoroughly. Pre-warm the ABC working solution at 37° C for 30 min before use. Use the working solution within 1 hour.
18. Upon completion of the 60 min incubation of **Step 16**, wash the plate 3 times with 0.3 ml TBS or PBS. For each wash, leave washing buffer in the wells for 1-2 min. Discard the washing buffer and blot the plate onto paper towels or other absorbent material.
19. Add 0.1 ml of prepared ABC Working Solution (prepared in **Step 17**) to each well and incubate the plate at 37° C for 30 min.
20. During the incubation period of **Step 19**, pre-warm TMB Color Developing Agent at 37° C for 30 min before use.
21. Upon completion of the 30 min incubation of **Step 19**, wash the plate 5 times with 0.3 ml TBS or PBS. For each wash, leave the washing buffer in the wells for 1-2 min. Discard the washing buffer and blot the plate onto paper towels or other absorbent material.
22. Add 0.1 ml of the TMB Stop Solution to each well. The acidic stop solution will change the mixture color to yellow. The yellow intensity is proportional to the amount of target protein captured by the plate.
23. Add 90 µl of the pre-warmed TMB Color Developing Agent into each well and incubate at 37° C for 18-22 min (shades of blue can be seen in the wells with the four most concentrated Protein Standard Solutions; the other control wells should show no obvious color).
24. Read the O.D. absorbance at 450nm in a microplate reader within 30 min after adding the stop solution. These readings are the O.D.450(Reading).

Calculating Protein Concentration

- For all wells, determine O.D.450(Relative):
  O.D.450(Relative) = O.D.450(Reading) – O.D.450(Blank)
- Plot the standard curve: plot O.D.450(Relative) of each standard solution (Y) vs. the respective concentration of the standard solution (X). See **Figure 1** for a typical standard curve.
- The mouse TP0 concentration of the samples can be interpolated from the standard curve. Multiply the interpolated concentration by the dilution factor to obtain the target protein concentration in the sample.