CELL APPLICATIONS, INC.

human TIE2 ELISA Kit

Cat. No. CL0519
96-wells

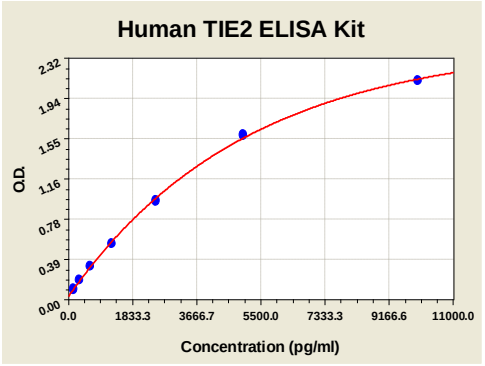
Target Protein Species:	Human
Range	156pg/ml – 10000pg/ml
Specificity	Cross-reactivates with human TIE1< 0.1%.

KIT COMPONENTS

Component	Amount
96-well plate precoated with anti-human TIE2 antibody	1 Plate
Protein Standard: Lyophilized recombinant human TIE2	2 tubes, 100 ng/tube
Sample Diluent Buffer	30 ml
Biotinylated Antibody (Anti-human TIE2)	130 µl (100x)
Antibody Diluent Buffer	12ml
Avidin-Biotin-Peroxidase Complex (ABC) Solution	130 µl (100x)
ABC Diluent Buffer	12 ml
Tetramethylbenzidine (TMB) Color Developing Agent	10 ml
TMB Stop Solution	10 ml

Storage

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



X	pg/ml	0.0	156	312	625	1250	2500	5000	10000
Y	O.D.450	0.039	0.114	0.195	0.330	0.546	0.959	1.592	2.114

Figure 1: TIE2 Standard Curve. Using the human TIE2 ELISA Kit, O.D. data was graphed against TIE2 protein concentration. The TMB reaction was incubated at 37° C for 10 min.

BACKGROUND

Tie2 is a receptor tyrosine kinase (RTK) with a unique extracellular ligand-binding domain comprised of immunoglobulin (IG) domains, epithelial growth factor (EGF) repeats, and fibronectin-like 3 (FN3) repeats. It is highly conserved among vertebrate species from zebrafish to human, with the highest homology in the kinase domain. Tie2 is expressed on endothelial cells (ECs) and hematopoietic stem cells (HSCs).[1] Tie2-/- mice die during embryogenesis at day 9.5 to 10.5; necropsy analyses have shown that the endothelial cells of such mice are present in normal numbers and assembled into tubes, but the vessels are immature and lack branch networks and hierarchical organization into large and small vessels. Observations in human subjects have revealed that deficient smooth muscle cell investment typical of venous malformations is associated with a mutation in the Tie2 RTK, suggesting that the Tie2 system may regulate the endothelial cell recruitment of stromal cells required to encase and thereby stabilize primitive endothelial tubes.[2]

The ligands of the Tie2 receptor have been identified as Ang1 and Ang2. Ang1 was identified as the major physiological ligand for Tie2, responsible for recruiting and sustaining periendothelial support cells. Ang2 was found to disrupt blood vessel formation in the developing embryo by antagonizing the effects of Ang1 and Tie2, and it was thus concluded that Ang2 represents a natural Ang1/Tie2 inhibitor. Extrapolation of these developmental findings to postnatal neovascularization has led to the dual inferences that Ang1 may induce maturation and stabilization of developing neovasculature, whereas Ang2 may cause destabilization required for additional sprout formation.[3] Ang1 binds to Tie2 and induces its activation via tyrosine phosphorylation. Through the phosphatidylinositol 3-kinase (PI3K)-Akt pathway and others, Ang1 exerts prosurvival, antipermeability, and anti-inflammatory effects on endothelial cells (ECs). Furthermore, it was demonstrated that Ang2 possesses both partial agonistic as well as antagonistic action on Tie2 in ECs—alone, Ang2 is a weak but necessary activator of Tie2, whereas in the presence of Ang1, Ang2 inhibits Tie2 signaling. Moreover, ECs secrete Ang2, which in turn maintains a basal level of Tie2 phosphorylation.[4]

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 Tetramethylbenzidine (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°C 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. Hayes, A.J. et al: Microvascu. Res. 58:224-37, 1999
2. Vikkula, M. et al: Cell 87:1181-90, 1996
3. Maisonpierre,P.C. et al: Science 277:55-60, 1997
4. Yuan, H.T. et al: Mol. Cell. Biol. 29:2011-22, 2009

ELISA PROTOCOL

Preparation of Test Samples

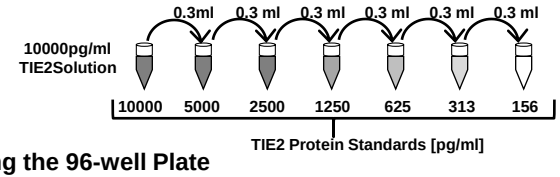
1. Process Test Samples in the following manner:
   - **Cell culture supernate, tissue lysate or body fluids**: Remove particulates by centrifugation.
   - **Serum**: Allow the serum to clot in a serum separator tube (about 2 hours) at room temperature. Centrifuge at approximately 1000 x g for 10 min. Analyze the serum immediately or aliquot and store frozen at -20° C.
   - **Plasma**: Collect plasma using heparin/EDTA/citrate as an anticoagulant. Centrifuge for 10 min at 1000 x g within 30 min of collection. Analyze 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 156-10000 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.
100-1000 ng/ml	1:100	1 µl	99 µl
10-100 ng/ml	1:10	10 µl	90 µl
156-10000 pg/ml	1:2	50 µl	50 µl
≤156 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 (156-10000 pg/ml)

4. Reconstitute the Lypophilized Recombinant Protein to make a 10.000 pg/ml human TIE2 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. Label 6 eppendorf tubes with the TIE2 protein concentrations to be prepared by serial dilution: 5000pg/ml, 2500pg/ml, 1250pg/ml, 625pg/ml, 313pg/ml, 156pg/ml.
6. Aliquot 0.3 ml of the Sample Diluent Buffer to the labeled tubes.
7. Serially dilute the protein standards into their respectively labeled tubes. Transfer 0.3 ml from the 10000pg/ml TIE2 Solution to the 5000pg/ml eppendorf tube and mix thoroughly. Transfer 0.3 ml of the 5000 pg/ml solution to the 2500pg/ml tube and mix thoroughly, Transfer 0.3 ml of the 2500pg/ml solution to the 1250pg/ml tube and mix, and so on to make the 625, 313 and 156 pg/ml solutions.
8. Store at 4° C until use.



Loading the 96-well Plate

9. 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.
10. Aliquot 0.1 ml of the standard solutions of the **Preparation of Standard Solutions** (156-10000pg/ml) into empty wells of the precoated 96-well plate. Duplicate measurements of standards are recommended.
11. 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.
12. Cover the 96-well plate and incubate at 37° C for 90 min.
13. During the **Step 12** 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.
14. Upon completion of the 90 min incubation of **Step 12**, 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.
15. Add 0.1 ml of the Biotinylated 1:100 Antibody Working Solution (prepared in **Step 13**) to each well and incubate the plate at 37° C for 60 min.
16. During the incubation period of **Step 15**, 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.
17. Upon completion of the 60 min incubation of **Step 15**, 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.
18. Add 0.1 ml of prepared ABC Working Solution (prepared in **Step 16**) to each well and incubate the plate at 37° C for 30 min.
19. During the incubation period of **Step 18**, pre-warm TMB Color Developing Agent at 37° C for 30 min before use.
20. Upon completion of the 30 min incubation of **Step 18**, 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.
21. Add 90 µl of the pre-warmed TMB Color Developing Agent into each well and incubate at 37° C for 10-15 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).
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. 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 human TIE2 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.