CELL APPLICATIONS, INC.

Human TGFbeta1 ELISA Kit

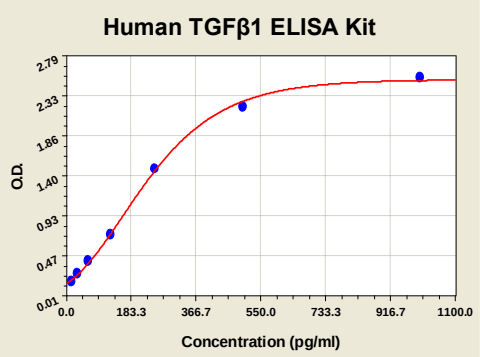
Cat. No. CL0513
96-wells

Target Protein Species: Human
Range 15.6 pg/ml – 1000pg/ml
Specificity Cross-reactivates with TGFβ2, TGFβ3, TGFβ5 <1%.

KIT COMPONENTS

Component	Amount
96-well plate precoated with anti-human TGFbeta1 antibody	1 Plate
Protein Standard: Lyophilized recombinant human TGFbeta1	2 tubes, 10 ng/tube
Sample Diluent Buffer	30 ml
Biotinylated Antibody (Anti-human TGFbeta1)	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	15.6	31.3	62.5	125	250	500	1000
Y	O.D.450	0.069	0.184	0.272	0.425	0.725	1.486	2.210	2.543

Figure 1: TGFbeta1 **Standard Curve.** Using the human TGFbeta1 ELISA Kit, O.D. data was graphed against TGFbeta1 protein concentration. The TMB reaction was incubated at 37° C for 12 min.

BACKGROUND

The TGF-beta superfamily of polypeptides comprises a group of highly conserved proteins, which includes various forms of TGF-beta, BMPs, Nodals, Activins, the anti-Müllerian hormone, and many other structurally related factors. They are ubiquitously expressed in eukaryotes and typically secreted into the extracellular milieu in an inactive form, where they become locally activated in response to the appropriate stimuli. These factors regulate cell migration, adhesion, multiplication, differentiation and death throughout the life span of the organism. The members of the TGF-beta family mediate such many different effects depending on the type and state of the cell.[1] Five TGF- beta have been cloned (TGF- beta 1–5). The distinguishing feature of the TGF- beta structure is the 'cysteine knot, ' formed from three of the four intramolecular disulfide bonds that maintains structural integrity for the monomer. TGF-beta signaling pathway is highly conserved from lower organisms to man. Although four receptors have been cloned (type I, II, III, endoglin), only two of them, the type II and type I receptors, have been conclusively proven to mediate TGF-beta signaling.[2] TGF-beta binds to the type II TGF-beta receptor (TbetaR-II) kinase. The type I receptor (TbetaR-I) is then recruited into the ligand/TbetaR-II complex and phosphorylated and activated by the TbetaR-II kinase. The activated TbetaR-I receptor then phosphorylates receptor-associated Smad2 and Smad3, which, in turn, form complexes with the common Smad, Smad4, and accumulate in the nucleus. In the nucleus, activated Smad complexes, along with co-activators and cell-specific DNA-binding factors, regulate gene expression and ultimately cell cycle and tissue repair. Besides this classical pathway, the TbetaR-II receptor is capable of partnering with other members of the type I receptor family, including Alk-1, Alk-2 and Alk-3. In these cases, TGF-beta signals can also activate the BMP Smads 1, 5 and 8. These alternate pathways normally appear to be restricted to certain cell- or tissue types. However, in the context of cancer, this second pathway can become constitutively activated and drive epithelial-to-mesenchymal transitions (EMT), cell motility and invasiveness, in which PI-3 kinase/Akt activation has been shown to be involved. However, many studies indicate that TGF-beta signaling can act either as a tumor promoter or a tumor suppressor.[3] TGFbeta elicits the tumor suppressor activity by potently inhibit the proliferation of epithelial, endothelial and haematopoietic cells. However, as tumors evolve, they often evade the TGFbeta growth inhibition due to mutational inactivation or dysregulated expression of various components of the TGFbeta signaling pathways or cell cycle regulatory network.[4]

References

1. Massagué, J. : Annu Rev Biochem. 67:753-91, 1998
2. Hu, P. P. et al: Endocrine Rev. 19:349-63, 1998
3. Wang, J. et al: Cancer Res. 68:3152-60, 2008
4. Massagué, J. et al: Cell 103:295-309, 2000

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.

ELISA PROTOCOL

Preparation of Test Samples

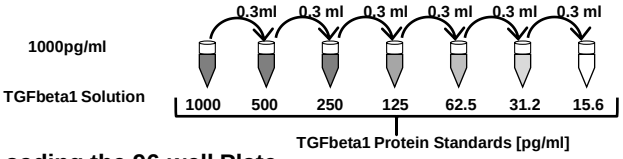
1. Process Test Samples in the following manner:
   - **Cell culture supernate, tissue lysate or body fluids**: Remove particulates by centrifugation. analyze immediately or aliquot and store at -20° C
   - **Serum**: Allow the serum to clot in a serum separator tube (about 30 min) at room temperature. Centrifuge at approximately 1000 x g for 10 min. Analyze the serum immediately or aliquot and store frozen at -70° C.
   - **Plasma:** Collect plasma using EDTA as an anticoagulant. Centrifuge for 15 min at 1000 x g within 30 min of collection. For eliminating platelet, suggesting that further centrifugation for 10 min at 2-8° C at 10000 x g. Analyze immediately or aliquot and store frozen at -70° C. Heparin and citrate are not recommended as the anticoagulant.
     Note: Bovine serum used in cell culture supernates may contain TGFβ1, avoiding using it.
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 15.6-1000 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.
10-100 ng/ml	1:100	1 µl	99 µl
1-10 ng/ml	1:10	10 µl	90 µl
15.6-1000 pg/ml	1:2	50 µl	50 µl
≤15.6pg/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 (15.6-1000 pg/ml)

4. Reconstitute the Lypophilized Recombinant Protein to make a 10.000 pg/ml human TGFbeta1 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 0.1 ml of the mixed 10,000 pg/ml TGFbeta1 solution to the eppendorf tube containing 0.9 ml diluent buffer and mix to make a 1000 pg/ml TGFbeta1 solution.
6. Label 6 eppendorf tubes with the human TGFbeta1 protein concentrations to be prepared by serial dilution :500pg/ml, 250pg/ml, 125pg/ml, 62.5pg/ml, 31.3pg/ml, 15.6pg/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 1000pg/ml TGFbeta1 Solution to the 500pg/ml eppendorf tube and mix thoroughly. Transfer 0.3 ml of the 500 pg/ml solution to the 250pg/ml tube and mix thoroughly, Transfer 0.3 ml of the 250pg/ml solution to the 125pg/ml tube and mix, and so on to make the 62.5, 31.3 and 15.6 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** (15.6-1000pg/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 90 µl of the pre-warmed TMB Color Developing Agent into each well and incubate at 37° C for 12-15 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).
23. 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.
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 human TGFbeta1 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.