

**Mouse CTLA4 ELISA Kit  
(mCTLA4-ELISA)**

*Cat. No. EK0717*

*96 Tests in 8 x 12 divisible strips*

**Background**

CTLA4 (Cytotoxic T-Lymphocyte Antigen 4), also known as CD152 (cluster of differentiation 152), is a receptor protein that down-regulates the immune system. CTLA4 is a member of the immunoglobulin superfamily, which is expressed on the surface of helper T cells and transmits an inhibitory signal to T cells. CTLA4 is similar to the T-cell co-stimulatory protein, CD28, and both molecules bind to CD80 and CD86, also called B7-1 and B7-2, respectively, on antigen-presenting cells. CTLA4 transmits an inhibitory signal to T cells, whereas CD28 transmits a stimulatory signal. Blockade of CTLA4 expression *in vitro* augments HIV-specific CD4-positive T-cell function.

ScienCell's mouse CTLA4 ELISA kit is based on standard sandwich enzyme-linked immunosorbent assay technology. Mouse CTLA4-specific polyclonal antibodies are pre-coated onto 8 x 12 divisible strips. The mouse-specific detection polyclonal antibodies are biotinylated. The test samples and biotinylated detection antibodies are subsequently added to the wells and then washed with PBS or TBS buffer. Avidin-Biotin-Peroxidase Complex is added and unbound conjugates are washed away with PBS or TBS buffer. HRP substrate TMB is used to visualize HRP enzymatic reaction. TMB is catalyzed by HRP to produce a blue color product that changes to yellow after adding acidic stop solution. The intensity of yellow is proportional to the amount of mouse CTLA4 in the sample that is captured on the strips.

<b>Size</b>	96 Tests in 8 x 12 divisible strips
<b>Assay type</b>	Sandwich ELISA
<b>Range</b>	156 pg/ml-10,000 pg/ml
<b>Sensitivity</b>	< 10 pg/ml
<b>Specificity</b>	Natural and recombinant mouse CTLA4
<b>Cross-reactivity</b>	No detectable cross-reactivity with other relevant proteins.
<b>Storage</b>	Store at 4°C for 6 months, at -20°C for 12 months. Avoid multiple freeze-thaw cycles.
<b>Shipping</b>	Shipped on gel ice.
<b>Application</b>	For quantitative detection of mouse CTLA4 in cell culture supernatants, serum and plasma (heparin).

- Kit components**
1. Lyophilized recombinant mouse CTLA4 standard: 10ng/tube×2.
  2. 8 x 12 divisible strips pre-coated with anti- mouse CTLA4 antibody.
  3. Sample diluent buffer: 30 ml
  4. Biotinylated anti-mouse CTLA4 antibody: 130µl, dilution 1:100.
  5. Antibody diluent buffer: 12ml.
  6. Avidin-Biotin-Peroxidase Complex (ABC): 130µl, dilution 1:100.
  7. TMB color developing agent: 10ml.
  8. TMB stop solution: 10ml.

**Materials** 1. Microplate reader.

**Required But** 2. Automated plate washer.

**Not Provided** 3. Adjustable pipettes and pipette tips. Multichannel pipettes are recommended for large amount of samples.

4. Clean tubes and Eppendorf tubes.

5. Washing buffer (neutral PBS or TBS).

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<sub>2</sub>O and adjust pH to 7.2-7.6. Finally, adjust the total volume to 1L.

Preparation of 0.01 M PBS: Add 8.5g NaCl, 1.4g Na<sub>2</sub>HPO<sub>4</sub> and 0.2g NaH<sub>2</sub>PO<sub>4</sub> to 1000ml distilled water and adjust pH to 7.2-7.6. Finally, adjust the total volume to 1L.

**Usage** This product is for research use only. It is not approved for use in humans, animals, or *in vitro* diagnostic procedures.

## Reference

1. Dariavach P, Mattéi MG, Golstein P, Lefranc MP (December 1988). "Human Ig superfamily CTLA-4 gene: chromosomal localization and identity of protein sequence between murine and human CTLA-4 cytoplasmic domains". Eur. J. Immunol. 18 (12): 1901–5.
2. Magistrelli G, Jeannin P, Herbault N, Benoit De Coignac A, Gauchat JF, Bonnefoy JY, Delneste Y (November 1999). "A soluble form of CTLA-4 generated by alternative splicing is expressed by nonstimulated human T cells". Eur. J. Immunol. 29 (11): 3596–602.
3. Waterhouse P, Penninger JM, Timms E, Wakeham A, Shahinian A, Lee KP, Thompson CB, Griesser H, Mak TW (November 1995). "Lymphoproliferative disorders with early lethality in mice deficient in Ctlα-4". Science 270(5238): 985–8.

## Protocol for Mouse CTLA4 ELISA (96-well format)

### Notes before you begin

1. To inspect the validity of experiment operation and the appropriateness of sample dilution proportion, a pilot experiment using standards and a small number of samples is recommended.
2. The TMB Color developing agent should be colorless and transparent before using.
3. Before using the kit, spin tubes and bring down all components to the bottom of tubes.
4. A duplicate well assay is recommended for both standard and samples.
5. Do not let wells dry, as this will inactivate active components in wells.
6. Do not reuse tips and tubes to avoid cross contamination.

7. Avoid using reagents from different batches.
8. In order to avoid marginal effect of plate incubation due to temperature difference (reaction may be stronger in the marginal wells), it is suggested that the diluted ABC and TMB solution be pre-warmed in 37°C for 30 minutes before use.

## **Preparation**

### **Sample Preparation and Storage**

Store samples to be assayed within 24 hours at 2-8°C. For long-term storage, aliquot and freeze samples at -20°C.

Avoid repeated freeze-thaw cycles.

- **Cell culture supernatants:** Remove particulates by centrifugation, assay immediately or aliquot and store at -20°C.
- **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 minutes. Analyze the serum immediately or aliquot and store samples at -20°C.
- **Plasma:** Collect plasma using heparin as an anticoagulant. Centrifuge for 15 minutes at 1500 x g within 30 minutes of collection. Assay immediately or aliquot and store samples at -20°C.

### **Sample Dilution Guideline**

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. Dilute the sample using the provided diluent buffer. The following is a guideline for sample dilution. Several trials may be necessary in practice. **The sample must be mixed well with the diluent buffer.**

- **High target protein concentration (100-1000 ng/ml).** The working dilution is 1:100. i.e. Add 1 µl sample into 99 µl sample diluent buffer.
- **Medium target protein concentration (10-100 ng/ml).** The working dilution is 1:10. i.e. Add 10 µl sample into 90 µl sample diluent buffer.
- **Low target protein concentration (156-10000 pg/ml).** The working dilution is 1:2. i.e. Add 50 µl sample to 50 µl sample diluent buffer.
- **Very Low target protein concentration ( $\leq 156$  pg/ml).** No dilution necessary, or the working dilution is 1:2.

### **Reagent Preparation and Storage**

A. Reconstitution of the mouse CTLA4 standard: CTLA4 standard solution should be prepared no more than 2 hours prior to the experiment. Two tubes of CTLA4 standard (10 ng per tube) are included in each kit. Use one tube for each experiment.

- 10,000 pg/ml of mouse CTLA4 standard solution: Add 1 ml sample diluent buffer into one tube, keep the tube at room temperature for 10 minutes and mix thoroughly.
- 5000pg/ml→156pg/ml of mouse CTLA4 standard solutions: Label 6 Eppendorf tubes with 5000 pg/ml, 2500 pg/ml, 1250 pg/ml, 625 pg/ml, 312 pg/ml, 156 pg/ml respectively. Aliquot 0.3ml of the sample diluent buffer into each tube. Add 0.3ml of the above 10,000pg/ml CTLA4 standard solution into 1st tube and mix. Transfer 0.3ml from 1st tube to 2nd tube and mix. Transfer 0.3ml from 2nd tube to 3rd tube and mix, and so on.

**Note:** The standard solutions are best used within 2 hours. The 10 ng/ml standard solution should be stored at 4°C for up to 12 hours, or at -20°C for up to 48 hours. Avoid repeated freeze-thaw cycles.

B. Preparation of biotinylated anti-mouse CTLA4 antibody working solution: The solution should be prepared no more than 2 hours prior to the experiment.

- The total volume should be: 0.1ml/well x (the number of wells). (Allowing 0.1-0.2 ml more than total volume).

- Biotinylated anti-mouse CTLA4 antibody should be diluted in 1:100 with the antibody diluent buffer and mixed thoroughly (i.e. Add 1µl Biotinylated anti-mouse CTLA4 antibody to 99µl antibody diluent buffer).
- C. Preparation of Avidin-Biotin-Peroxidase Complex (ABC) working solution: The solution should be prepared no more than 1 hour prior to the experiment.
- The total volume should be: 0.1ml/well x (the number of wells). (Allowing 0.1-0.2 ml more than total volume).
  - Avidin-Biotin-Peroxidase Complex (ABC) should be diluted in 1:100 with the ABC dilution buffer and mixed thoroughly (i.e. Add 1µl ABC to 99µl ABC diluent buffer).

### **Assay Procedure**

The ABC working solution and TMB color developing agent must be kept warm at 37°C for 30 minutes before use. When diluting samples and reagents, they must be mixed completely and evenly. Standard CTLA4 detection curve should be prepared for each experiment. The user will decide sample dilution fold by crude estimation of CTLA4 amount in samples.

1. Aliquot 0.1ml per well of the 10,000pg/ml, 5000pg/ml, 2500pg/ml, 1250pg/ml, 625pg/ml, 312pg/ml, 156pg/ml mouse CTLA4 standard solutions into the pre-coated 8 x 12 divisible strips. Add 0.1ml of the sample diluent buffer into the control well (**blank well**). Add 0.1ml of each properly diluted sample of mouse cell culture supernatants, serum or plasma (heparin) to each empty well. See “**Sample Dilution Guideline**” above for details. We recommend that each mouse CTLA4 standard solution and each sample is measured in duplicate.
2. Seal the strips with the cover and incubate at 37°C for 90 minutes.
3. Remove the cover, discard the strips’ contents, and blot the strips onto paper towels or other absorbent material. **Do NOT** let the wells completely dry at any time.
4. Add 0.1ml of biotinylated anti-mouse CTLA4 antibody working solution into each well and incubate the strips at 37°C for 60 minutes.
5. Wash the strips 3 times with 0.01M TBS or 0.01M PBS, and each time let washing buffer stay in the wells for 1 minute. Discard the washing buffer and blot the strips onto paper towels or other absorbent material. (**Strips Washing Method**: Discard the solution in the wells without touching the side walls. Blot the strips onto paper towels or other absorbent material. Soak each well with at least 0.3 ml PBS or TBS buffer for 1~2 minutes. Repeat this process two additional times for a total of THREE washes. Note: For automated washing, aspirate all wells and wash THREE times with PBS or TBS buffer, overfilling wells with PBS or TBS buffer. Blot the strips onto paper towels or other absorbent material).
6. Add 0.1ml of prepared ABC working solution into each well and incubate the strips at 37°C for 30 minutes.
7. Wash the strips 5 times with 0.01M TBS or 0.01M PBS, and each time let washing buffer stay in the wells for 1-2 minutes. Discard the washing buffer and blot the strips onto paper towels or other absorbent material. (See Step 5 for strip washing method).
8. Add 90 µl of prepared TMB color developing agent into each well and incubate the strips at 37°C in dark for 20-25 minutes (**Note**: For reference only, the optimal incubation time should be determined by end user. And the shades of blue can be seen in the wells with the four most concentrated mouse CTLA4 standard solutions; the other wells show no obvious color).
9. Add 0.1ml of prepared TMB stop solution into each well. The color changes to yellow immediately.
10. Read the O.D. absorbance at 450 nm in a microplate reader within 30 minutes after adding the stop solution.

For calculation, (the relative O.D.450) = (the O.D.450 of each well) – (the O.D.450 of blank 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). The mouse CTLA4 concentration of the samples can be interpolated from the standard curve.

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

## **Summary**

1. Add samples and standards and incubate the strips at 37°C for 90 minutes. Do not wash.
2. Add biotinylated antibodies and incubate the strips at 37°C for 60 minutes. Wash strips 3 times with 0.01M TBS.
3. Add ABC working solution and incubate the strips at 37°C for 30 minutes. Wash strips 5 times with 0.01M TBS.
4. Add TMB color developing agent and incubate the strips at 37°C in dark for 20-25 minutes.
5. Add TMB stop solution and read.

## **Typical Data Obtained from Mouse CTLA4**

(TMB reaction incubate at 37°C for 20 minutes)

Concentration (pg/ml)	0.0	156	312	625	1250	2500	5000	10000
Absorbance (450 nm)	0.045	0.056	0.072	0.109	0.221	0.496	1.004	1.665

## **Typical Mouse CTLA4 ELISA Kit Standard Curve**

This standard curve was generated for demonstration purpose only. A standard curve must be run with each assay.

