



LEGEND MAX[™]
ELISA Kit



Human Soluble CTLA-4

Cat. No. 437407

ELISA Kit for Accurate Quantitation of Human Soluble
CTLA-4 from Cell Culture Supernatant, Serum, Plasma and
Other Biological Fluids

BioLegend, Inc.
biolegend.com

It is highly recommended that this manual be read in its entirety before using this product. Do not use this kit beyond the expiration date.

For Research Purposes Only. Not for use in diagnostic or therapeutic procedures. Purchase does not include or carry the right to resell or transfer this product either as a stand-alone product or as a component of another product. Any use of this product other than the permitted use without the express written authorization of BioLegend is strictly prohibited.



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LEGEND MAX™ Human Soluble CTLA-4 ELISA Kit

Introduction:

CTLA-4 (Cytotoxic T-Lymphocyte Antigen 4), also known as CD152, is a protein that acts as an important regulator of the immune system. CTLA-4 is expressed on the surface of Helper T cells and transmits an inhibitory signal to T cells. Mutations in the CTLA-4 gene have been associated with various autoimmune diseases, such as systemic lupus erythematosus and insulin-dependent diabetes mellitus.

A novel transcript of the CTLA-4 gene was found to represent a native soluble form of CTLA-4 (sCTLA-4). A subsequent study showed that some patients with autoimmune thyroid disease (ATD) had higher concentrations of sCTLA-4 when compared to apparently healthy volunteers. Moreover, the immunoreactivity of sCTLA-4 was inhibited by its binding to B7.1, suggesting that sCTLA-4 is a functional receptor. Soluble CTLA-4 also plays a role in the initial immune response to infection of immune cells by HIV by way of the CD-1 pathway.

The BioLegend LEGEND MAX™ Human Soluble CTLA-4 ELISA kit is a Sandwich Enzyme-Linked Immunosorbent Assay (ELISA) with a 96-well strip plate that is pre-coated with a monoclonal mouse anti-human sCTLA-4 capture antibody. The detection antibody is a biotinylated monoclonal mouse anti-human sCTLA-4 antibody. This kit is specifically designed for the accurate quantitation of sCTLA-4 from cell culture supernatant, serum, plasma and other biological fluids. This kit is analytically validated with ready-to-use reagents.

Materials Provided:

Description	Quantity	Volume (per bottle)	Part #
Anti-Human sCTLA-4 Pre-coated 96-well Strip Microplate	1 plate		79347
Human sCTLA-4 Detection Antibody	1 bottle	12 mL	79349
Human sCTLA-4 Standard	1 vial	lyophilized	79348
Matrix C (for serum and plasma samples only)	1 vial	lyophilized	78316
Avidin-HRP A	1 bottle	12 mL	79131
Assay Buffer A	1 bottle	25 mL	78232
Wash Buffer (20X)	1 bottle	50 mL	78233
Substrate Solution F	1 bottle	12 mL	79132
Stop Solution	1 bottle	12 mL	79133
Plate Sealers	4 sheets		78101

Materials to be Provided by the End-User:

- Microplate reader able to measure absorbance at 450 nm
- Adjustable pipettes to measure volumes ranging from 1 μ L to 1,000 μ L
- Deionized water
- Wash bottle or automated microplate washer
- Log-Log graph paper or software for data analysis
- Tubes to prepare standard dilutions
- Timer
- Plate Shaker
- Polypropylene vials

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Storage Information:

Store unopened kit components between 2°C and 8°C. Do not use this kit beyond its expiration date.

Opened or Reconstituted Components	
Microplate wells	If not all microplate strips are used, remove the excess strips by pressing up from underneath each strip. Place excess strips back in the foil pouch with the included desiccant pack and reseal. Store between 2°C and 8°C for up to one month.
Standard	The remaining reconstituted standard stock solution and Matrix C can be aliquoted into polypropylene vials and stored at -70°C for up to one month. Avoid repeated freeze-thaw cycles.
Matrix C	
Detection Antibody	Store opened reagents between 2°C and 8°C and use within one month.
Avidin-HRP A	
Assay Buffer A	
Wash Buffer (20X)	
Substrate Solution F	
Stop Solution	

Health Hazard Warnings:

1. Reagents that contain preservatives may be harmful if ingested, inhaled or absorbed through the skin. Refer to the MSDS online at BioLegend's website for details (www.biolegend.com/msds).
2. Substrate Solution F is harmful if inhaled or ingested. Avoid skin, eye and clothing contact.
3. To reduce the likelihood of blood-borne transmission of infectious agents, handle all serum, plasma and other biological fluids in accordance with NCCLS regulations.
4. Stop Solution contains strong acid. *Wear eye, hand, and face protection.*
5. Before disposing of the plate, rinse it with an excess amount of tap water.

Specimen Collection and Handling:

Specimens should be clear and non-hemolyzed. If possible, unknown samples should be run at a number of dilutions to determine the optimal dilution factor that will ensure accurate quantitation.

Cell Culture Supernatant: If necessary, centrifuge all samples to remove debris prior to analysis. It is recommended that samples be stored at $< -70^{\circ}\text{C}$. Avoid repeated freeze-thaw cycles.

Serum: Use a serum separator tube and allow clotting for at least 30 minutes, then centrifuge for 10 minutes at $1,000 \times g$. Remove serum layer and assay immediately or store serum samples at $< -70^{\circ}\text{C}$. Avoid repeated freeze-thaw cycles.

Plasma: Collect blood samples in citrate, heparin or EDTA containing tubes. Centrifuge for 10 minutes at $1,000 \times g$ within 30 minutes of collection. Assay immediately or store plasma samples at $< -70^{\circ}\text{C}$. Avoid repeated freeze-thaw cycles.

Reagent and Sample Preparation:

Note: All reagents should be diluted immediately prior to use.

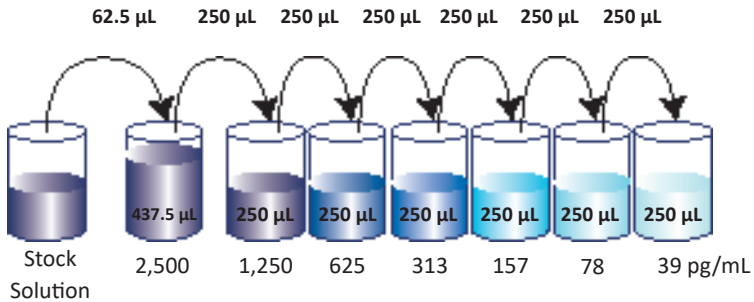
1. Dilute the 20X Wash Buffer to 1X with deionized water. For example, make 1 liter of 1X Wash Buffer by adding 50 mL of 20X Wash Buffer to 950 mL of deionized water. If crystals have formed in the 20X Wash Buffer, bring to room temperature and vortex until dissolved.
2. Reconstitute the lyophilized Human sCTLA-4 Standard by adding the volume of Assay Buffer A to make the 20 ng/mL standard stock solution (Refer to LEGEND MAX Kit Lot-Specific Certificate of Analysis/LEGEND MAX Kit Protocol). Allow the reconstituted standard to sit at room temperature for 15-20 minutes, then briefly vortex to mix completely.
3. If serum or plasma samples will be assayed, reconstitute the lyophilized Matrix C by dispensing 2 mL of deionized water into the vial and allow the reconstituted Matrix C to sit at room temperature for 15-20 minutes, then vortex to mix completely.
4. In general, cell culture supernatant samples are analyzed without dilutions. However, if dilutions are required, use Assay Buffer A as the sample diluent.
5. In general, serum and plasma samples are analyzed without dilutions. However, if dilutions are required, use Matrix C as the sample diluent.

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Assay Procedure:

Note: Do not mix reagents from different kits or lots. Reagents and/or antibodies from different manufacturers should not be used with this kit.

1. Bring all reagents to room temperature prior to use. It is strongly recommended that all standards and samples be run in duplicate or triplicate. A standard curve is required for each assay.
2. If not all microplate strips will be used, remove the excess strips by pressing up from underneath each strip. Place excess strips back in the foil pouch with the included desiccant pack and reseal.
3. Prepare 500 μL of the 2,500 pg/mL top standard by diluting 62.5 μL of the standard stock solution in 437.5 μL of Assay Buffer A. Perform six two-fold serial dilutions of the 2,500 pg/mL top standard in separate tubes using Assay Buffer A as the diluent. Thus, the Human sCTLA-4 Standard concentrations in the tubes are 2,500 pg/mL , 1,250 pg/mL , 625 pg/mL , 313 pg/mL , 157 pg/mL , 78 pg/mL , and 39 pg/mL , respectively. Assay Buffer A serves as the zero standard (0 pg/mL).



4. Wash the plate 4 times with at least 300 μL of 1X Wash Buffer per well and blot any residual buffer by firmly tapping plate upside down on absorbent paper. All subsequent washes should be performed similarly.
5. **For measuring samples of cell culture supernatant:**
 - a) Add 50 μL of Assay Buffer A to each well that will contain either standard dilutions or samples.
 - b) Add 50 μL of standard dilutions or samples to the appropriate wells.

For measuring serum or plasma samples:

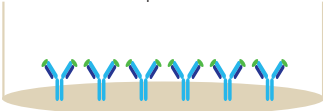
- a) Add 50 μL of Matrix C to each well that will contain the standard dilutions. Add 50 μL of Assay Buffer A to each well that will contain samples.

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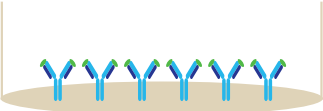
- b) Add 50 μ L of standard dilutions to the wells containing Matrix
 - c) Add 50 μ L of diluted serum or plasma samples to the wells containing Assay Buffer A.
6. Seal the plate with a Plate Sealer included in the kit and incubate the plate at room temperature for 2 hours while shaking at 200 rpm.
7. Discard the contents of the plate into a sink, then wash the plate 4 times with 1X Wash Buffer as in step 4.
8. Add 100 μ L of the Human sCTLA-4 Detection Antibody solution to each well, seal the plate and incubate at room temperature for 1 hour while shaking.
9. Discard the contents of the plate into a sink, then wash the plate 4 times with 1X Wash Buffer as in step 4.
10. Add 100 μ L of Avidin-HRP A solution to each well, seal the plate and incubate at room temperature for 30 minutes while shaking.
11. Discard the contents of the plate into a sink, then wash the plate 5 times with 1X Wash Buffer as in step 4. For this final wash, soak wells in 1X Wash Buffer for 30 seconds to 1 minute for each wash. This will help minimize background.
12. Add 100 μ L of Substrate Solution F to each well and incubate for 25 minutes in the dark. Wells containing human sCTLA-4 should turn blue in color with an intensity proportional to its concentration. It is not necessary to seal the plate during this step.
13. Stop the reaction by adding 100 μ L of Stop Solution to each well. The solution color should change from blue to yellow.
14. Read absorbance at 450 nm within 30 minutes. If the reader is capable of reading at 570 nm, the absorbance at 570 nm can be subtracted from the absorbance at 450 nm.

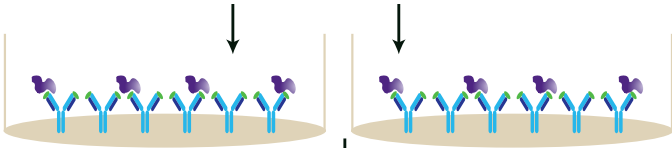
Assay Procedure Summary

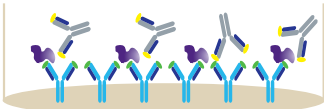
- For measuring cell culture supernatant:**
Wash 4 times
Add 50 μ L Assay Buffer A to standard and sample wells

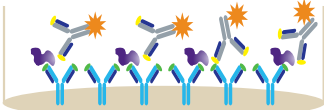


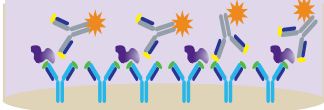
For measuring serum or plasma:
Wash 4 times
Add 50 μ L Matrix C to standard wells
Add 50 μ L Assay Buffer A to sample wells

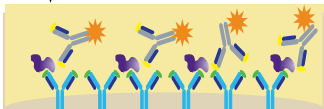

- Add 50 μ L diluted standards to standard wells
Add 50 μ L samples to sample wells
Incubate 2 hr, RT, shaking


- Wash 4 times
Add 100 μ L Detection Antibody solution
Incubate 1 hr, RT, shaking


- Wash 4 times
Add 100 μ L Avidin-HRP A solution
Incubate 30 min, RT, shaking


- Wash 5 times
Add 100 μ L Substrate Solution F
Incubate 25 min, RT, in the dark


- Add 100 μ L Stop Solution


- Read absorbance at 450 nm and 570 nm

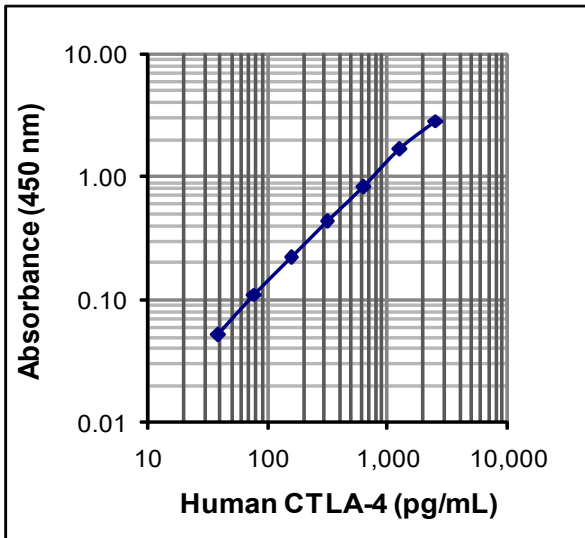
Calculation of Results:

The data can be best calculated with computer-based curve-fitting software using a 5- or 4-parameter logistics curve-fitting algorithm. If an appropriate software is not available, use log-log graph paper to determine sample concentrations. Determine the mean absorbance for each set of duplicate or triplicate standards, controls, and samples. Plot the standard curve on log-log graph paper with cytokine concentration on the X-axis and absorbance on the Y-axis. Draw a best fit line through the standard points. To determine the unknown cytokine concentrations, find the mean absorbance value of the unknown concentration on the Y-axis and draw a horizontal line to the standard curve. At the point of intersection, draw a vertical line to the X-axis and read the cytokine concentration.

If samples were diluted, multiply the concentration by the appropriate dilution factor. If a test sample's absorbance value falls outside the linear portion of the standard curve, the test sample needs to be re-analyzed at a higher (or lower) dilution as appropriate.

Typical Data:

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



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Performance Characteristics:

Specificity: No cross reactivity was observed when this kit was used to analyze 18 human recombinant cytokines/chemokines, each at a concentration of 50 ng/mL.

IL-1 α , IL-1 β , IL-2, IL-6, IL-8, IL-10, IL-11, IL-13, IL-15, IL-17A, IL-17A/F, IL-22, IL-27, IL-32 α , FGF-basic, IFN- γ , TNF- α , TSLP

Sensitivity: The minimum detectable concentration of sCTLA-4 is 17 pg/mL.

Recovery: Soluble CTLA-4 at concentrations of 1,250 pg/mL, 625 pg/mL, and 313 pg/mL, was spiked into 5 human serum samples, and then analyzed with the LEGEND MAX™ Human Soluble CTLA-4 ELISA kit. On average, 102.2% of the protein was recovered from the serum samples.

Linearity: Five human serum samples containing high concentrations of sCTLA-4 were diluted with Assay Buffer A to produce samples with values within the dynamic range and then assayed with the kit to determine the linearity of dilution. On average, 109% of the expected protein was recovered from serum samples.

Intra-Assay Precision: Two samples containing different sCTLA-4 concentrations were tested with eight replicates in one assay.

Concentration	Sample 1	Sample 2
Number of Replicates	8	8
Mean Concentration (pg/mL)	625	298
Standard Deviation	8.2	11.0
% CV	1.3	3.7

Inter-Assay Precision: Two samples containing different concentrations of human sCTLA-4 were analyzed in three independent assays.

Concentration	Sample 1	Sample 2
Number of Assays	3	3
Mean Concentration (pg/mL)	579	289
Standard Deviation	28.4	22.1
% CV	4.9	7.6

Normal Serum Ranges: Twenty-two normal human serum samples were tested and none of them had detectable sCTLA-4.

Troubleshooting Guide:

Problem	Probable Cause	Solution
High Background	Background wells were contaminated	Avoid cross-well contamination by using the provided plate sealers. Use multichannel pipettes and change tips between pipetting samples and reagents.
	Insufficient washes	Increase number of washes. Increase soaking time between washes prior to addition of substrate solution.
	TMB Substrate Solution was contaminated	TMB Substrate Solution should be clear and colorless prior to addition to wells. Use a clean container prior to pipetting substrate solution into wells.
No or poor signal	Detection Antibody, Avidin-HRP or Substrate solution were NOT added	Rerun the assay and follow the protocol.
	Wrong reagent or reagents were added in wrong sequential order	
	Insufficient plate agitation	The plate should be agitated during all incubation steps using a plate shaker at a speed where solutions in wells are within constant motion without splashing.
	The wash buffer contains Sodium Azide (NaN ₃)	Avoid Sodium Azide contamination in the wash buffer as it inhibits HRP activity.
	Incubations were done at an inappropriate temperature, timing or without agitation	Rerun the assay and follow the protocol.
Low or poor standard curve signal	The standard was incorrectly reconstituted or diluted	Adjust the calculations and follow the protocol.
	Standard was inappropriately stored	Store the reconstituted standard stock solution in polypropylene vials at -70°C. Avoid repeated freeze-thaw cycles.
	Reagents added to wells with incorrect concentrations	Check for pipetting errors and the correct reagent volume.

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Problem	Probable Cause	Solution
Signal is high, standard curves have saturated signal	Standard reconstituted with less volume than required	Reconstitute new lyophilized standard with the correct volume of solution recommended in the protocol.
	Standards/samples, detection antibody, Avidin-HRP or substrate solution were incubated for too long	Rerun the assay and follow the protocol.
Sample readings are out of range	Samples contain no or below detectable levels of the analyte	If samples are below detectable levels, it may be possible to use a larger sample volume. Contact technical support for appropriate protocol modifications.
	Samples contain analyte concentrations greater than highest standard point	Samples may require dilution and analysis.
High variation in samples and/or standards	Multichannel pipette errors	Confirm that pipette calibrations are accurate.
	Plate washing was not adequate or uniform	Ensure pipette tips are tightly secured. Ensure uniformity in all wash steps.
	Non-homogenous samples	Thoroughly mix samples before assaying.
	Samples may have high particulate matter	Remove particulate matter by centrifugation.
	Cross-well contamination	Do not reuse plate sealers. Always change tips for reagent additions. Ensure that pipette tips do not touch the reagents on the plate.

ELISA Plate Template

	1	2	3	4	5	6	7	8	9	10	11	12
A												
B												
C												
D												
E												
F												
G												
H												



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