BioLegend®

Enabling Legendary Discovery™

LEGEND MAX™

ELISA Kit

Mouse Latent TGF-β

Cat. No. 433007

ELISA Kit for Accurate Quantitation of Mouse Latent TGF-β from Cell Culture Supernatant, Serum, Plasma and Other Biological Fluids

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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.

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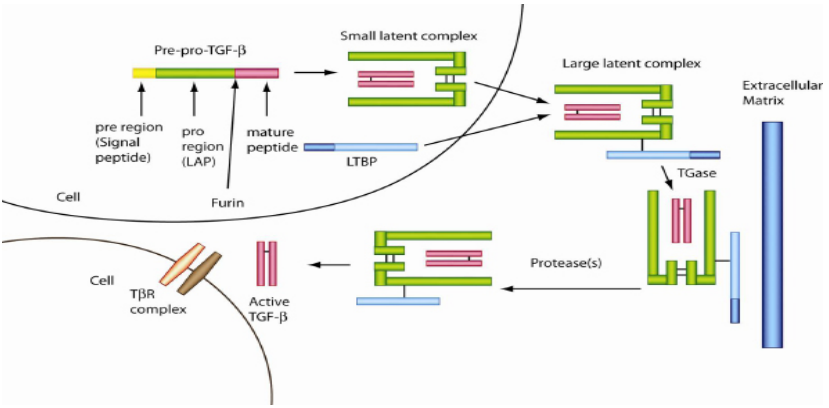
Introduction:

Latent TGF-β is secreted as a complex consisting of LAP and TGF-β. LAP and TGF-β are synthesized as a single propeptide precursor of 390 amino acids with an N-terminal signal peptide of 29 amino acids, a 249 amino acids pro-region (LAP), and a 112 amino acids C-terminal region (TGF-β).

Both LAP and TGF-β exist as homodimers in circulation, but the disulfide linked homodimers of LAP and TGF-β remain non-covalently associated, forming the small latent TGF-β complex (SLC, 100 kDa). The large latent TGF- β complex (LLC, 235 – 260 kDa) contains a third component, the latent TGF- β binding protein (LTBP), which is linked to LAP by a single disulfide bond. LTBP does not confer latency but is for efficient secretion of the complex to extracellular sites. The following picture illustrates the relationships among LAP, TGF-β, latent TGF- β and LTBP.



TGF-β and LAP can be released (activated) by many factors including enzymes and low or high pH. TGF-β and LAP have multiple function such as regulating proliferation and differentiation of various cell types.

Increased production and activation of latent TGF-β have been linked to immune defects associated with malignancy and autoimmune disorders, to susceptibility to opportunistic infection, and to the fibrotic complications associated with chronic inflammatory conditions.

Latent TGF-β has been found to portect glomerulonephritis. It is a surface marker of activated regulatory T cells.

LEGEND MAX™ Mouse Latent TGF-β 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-mouse Latent TGF-β capture antibody. The detection antibody is a biotinylated monoclonal mouse anti-mouse Latent TGF-β antibody. This kit is specifically designed for the accurate quantitation of both the small and large latent TGF-β from cell culture supernatant, serum, plasma and other biological fluids. This kit is analytically validated with ready-to-use reagents.

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Materials Provided:

Description	Quantity	Volume (per bottle)	Part #
Anti- Mouse Latent TGF-β Pre-coated 96-well Strip Microplate	1 plate		79717
Mouse Latent TGF-β Detection Antibody	1 bottle	12 mL	79718
Mouse Latent TGF-β Standard	1 vial	lyophilized	79720
Avidin-HRP D	1 bottle	12 mL	78237
Assay Buffer E	1 bottle	30 mL	79559
Wash Buffer (20X)	1 bottle	50 mL	78233
Substrate Solution D	1 bottle	12 mL	78115
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 µL to 1,000 µL
- 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

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 can be aliquoted into polypropylene vials and stored at -70°C for up to one month. Avoid repeated freeze-thaw cycles.
Detection Antibody	Store opened reagents between 2°C and 8°C and use within one month.
Avidin-HRP D	
Assay Buffer E	
Wash Buffer (20X)	
Substrate Solution D	
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 D 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.

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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°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 x g. Remove serum layer and assay immediately or store serum samples at < -70°C. Avoid repeated freeze-thaw cycles.

Plasma: Collect blood samples in a citrate, heparin or EDTA containing tube. Centrifuge for 10 minutes at 1,000 x g within 30 minutes of collection. Assay immediately or store plasma samples at < -70°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.
2. Reconstitute the lyophilized Mouse Latent TGF-β Standard by adding the volume of Assay Buffer E indicated 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. Serum and plasma samples must be diluted 1:50 with Assay Buffer E prior to sample analysis, e.g. add 4 µL of sample to 196 µL Assay Buffer E in a polypropylene microfuge tube. If further dilution is required, the sample should be diluted in Assay Buffer E.
4. In general, cell culture supernatant samples are analyzed without dilutions. However, if dilutions are required, samples should be diluted in Assay Buffer E.

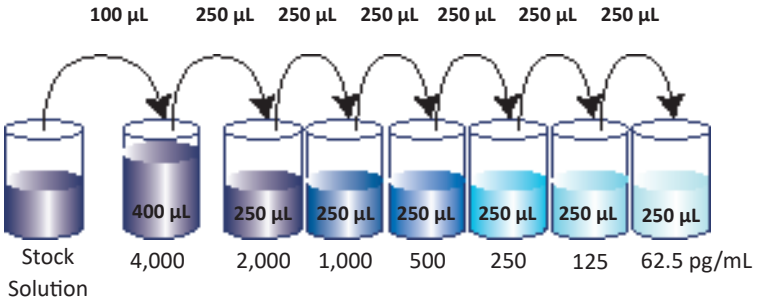
Assay Procedure:

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. 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 4,000 pg/mL top standard by diluting 100 µL of the 20 ng/mL standard stock solution into 400 µL of Assay Buffer E in a polypropylene microfuge tube. Starting with the 4,000 pg/mL top standard, perform six two-fold serial dilutions by mixing and then transferring 250 µL to the next polypropylene microfuge tube. The final mouse latent TGF-β standard concentrations in the tubes are 4,000 pg/mL, 2,000 pg/mL, 1,000 pg/mL, 500 pg/mL, 250 pg/mL, 125 pg/mL, and 62.5 pg/mL, respectively. Assay Buffer E 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 the plate upside down on absorbent paper. All subsequent washes should be performed similarly.

5. Add 50 µL of Assay Buffer E to each well that will contain either standard dilutions or samples.

6. Add 50 µL of standard dilutions or samples to the appropriate wells.

7. 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.

8. Discard the contents of the plate into a sink, then wash the plate 4 times with 1X Wash Buffer as in step 4.

9. Add 100 µL of Mouse Latent TGF-β Detection Antibody solution to each well, seal the plate and incubate at room temperature for 1 hour while shaking.

10. Discard the contents of the plate into a sink, then wash the plate 4 times with 1X Wash Buffer as in step 4.

11. Add 100 µL of Avidin-HRP D solution to each well, seal the plate and incubate at room temperature for 30 minutes while shaking.

12. 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.

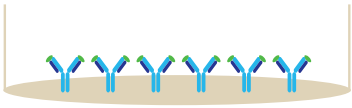
13. Add 100 µL of Substrate Solution D to each well and incubate for 20 minutes in the dark. Wells containing Mouse latent TGF-β should turn blue in color with an intensity proportional to its concentration. It is not necessary to seal the plate during this step.

14. Stop the reaction by adding 100 µL of Stop Solution to each well. The solution color should change from blue to yellow.

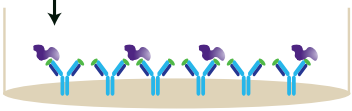
15. Read absorbance at 450 nm immediately. If the reader is capable of reading at 570 nm, the absorbance at 570 nm can be subtracted from the absorbance at 450 nm.

Assay Procedure Summary

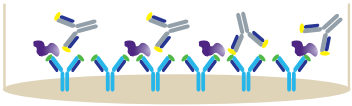
1. Wash 4 times
Add 50 µL Assay Buffer E



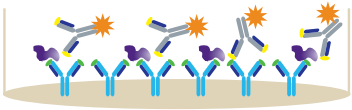
2. Add 50 µL diluted standards or samples
Incubate 2 hrs, RT, shaking



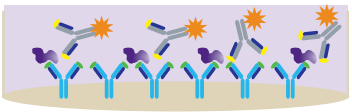
3. Wash 4 times
Add 100 µL Detection Antibody solution
Incubate 1 hr, RT, shaking



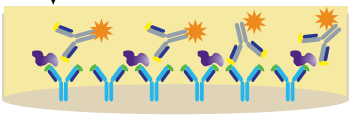
4. Wash 4 times
Add 100 µL Avidin-HRP D solution
Incubate 30 min, RT, shaking



5. Wash 5 times
Add 100 µL Substrate Solution D
Incubate 20 min, RT, in the dark



6. Add 100 µL Stop Solution



7. Read absorbance at 450 nm and 570 nm

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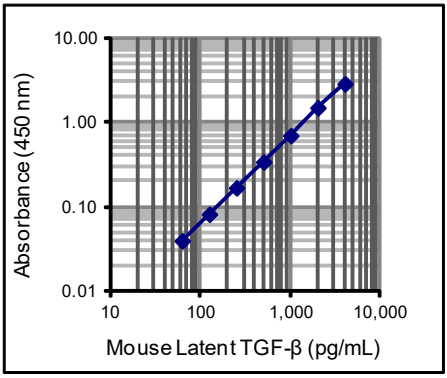
Calculation of Results:

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.



Performance Characteristics:

Specificity:

This kit detects small and large latent TGF-β in mouse samples.

No cross-reactivity was observed when this kit was used to analyze the following recombinant cytokines/chemokines at up to 50~100 ng/mL:

Mouse	IL-2, IL-4, IL-25, IL-6, IL-10, IL-12p70, IL-17A, IL-27, GM-CSF, MCP-1, TNFα, IFNγ
Human	TGF-β1, LAP, Latent TGF-β, IL-4, IL-5, IL-17A, IL-27, TNFα, IFNγ
Rat	TNFα, IFNγ

Sensitivity: The average minimum detectable concentration is 15.6 pg/mL.

Recovery: Recombinant mouse latent TGF-β (2,000, 500 and 125 pg/mL) was spiked into BALB/c, C57 BL/6, CD-1, Swiss Webster serum samples (1:50 diluted) and analyzed with LEGEND MAX™ Mouse Latent TGF-β ELISA kit. On average, 128 % of latent TGF-β was recoved from serum samples.

Linearity: BALB/C, C57BL/6, CD-1 and Swiss Webster mouse serum were diluted 1:50, 1:100, 1:200 and 1:400 with Assay Buffer E, and then assayed. On average, 77 % of expected latent TGF-β was detected from serum samples.

Intra-Assay Precision: Two samples containing different latent TGF-β concentrations were tested on one plate with 16 replicates.

Concentration	Sample 1	Sample 2
Number of Replicates	16	16
Mean Concentration (pg/mL)	529.1	2222.2
Standard Deviation	15.7	47.2
% CV	3.0	2.1

Inter-Assay Precision: Two samples containing different concentrations of latent TGF-β were tested in four independent assays.

Concentration	Sample 1	Sample 2
Number of Assays	3	3
Mean Concentration (pg/mL)	478.3	2065.2
Standard Deviation	41.2	100.6
% CV	8.6	4.9

Biological Samples:

Mouse Serum Samples

Mouse Strain	BALB/c	C57BL/6	CD-1	Swiss Webster
Pooled Serum (ng/mL)	46.9	138.2	65.4	64.9

Cell Culture Supernatant: - Mouse splenocytes at a density of $2x10^6$ cells/mL were stimulated with CD3/CD28 at 37°C for 2 days. The cell culture supernatants were collected and assayed for the concentration of natural mouse latent TGF-β.

sample	concentration (pg/mL)
CD3/CD28	476
Unstimulated	below the lowest point of 62.5 pg/mL

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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 (NaN3)	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.

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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433007-R08