



**LEGEND MAX<sup>™</sup>**  
ELISA Kit



**Human Granzyme B**

Cat. No. 439207

ELISA Kit for Accurate Quantitation of Human  
Granzyme B from Cell Culture Supernatant, Serum,  
Plasma, and Other Biological Fluids

BioLegend, Inc.  
[biolegend.com](http://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.***

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# LEGEND MAX™ Human Granzyme B ELISA Kit

## Introduction:

Granzymes are serine proteases that are released by cytoplasmic granules within cytotoxic T cells and natural killer cells. Human Granzyme B is one of the five members of the human granzyme family which includes Granzymes A, B, G, H, and K. Granzyme B is also known as Granzyme 2 or serine protease B (CSP-B for cytotoxic serine protease B) or CCP1 (cytotoxic cell protease-1). It is expressed as a 32 kD protein encoded by the GZMB gene. While Granzyme B is mostly expressed by CTL (Cytotoxic T Lymphocyte) and NK cells, it is also expressed in resting and activated plasmacytoid dendritic cells. Much lower levels are found in monocytes, resting T-cells, B-cells, activated granulocytes, and activated monocyte-derived dendritic cells.

Granzyme B is crucial for the rapid induction of target cell apoptosis by CTL in cell-mediated immune response. Granzyme B and perforin have been shown to induce CTL-mediated target cell DNA fragmentation and apoptosis. Once released from the CTL, Granzyme B binds its receptor, the mannose-6-phosphate/insulin-like growth factor II receptor, and is endocytosed but remains arrested in endocytic vesicles until released by perforin. Once in the cytosol, Granzyme B targets caspase-3 and other caspases directly or indirectly through the mitochondria, initiating the caspase cascade which leads to DNA fragmentation and apoptosis.

Elevated Granzyme B levels have been linked with various disease processes. For example, increased levels of soluble granzymes have been found in patients with systemic viral infections. It is also shown that the presence of a high percentage of Granzyme B positive CTLs in glands of patients suffering from Hodgkin's disease correlate with a severe prognosis. Synovial fluid from rheumatoid arthritis patients contains elevated soluble Granzyme A and B levels compared with healthy donors and patients with osteoarthritis.

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

**Materials Provided:**

Description	Quantity	Volume (per bottle)	Part #
Anti-Human Granzyme B Pre-coated 96-well Strip Microplate	1 plate		79798
Human Granzyme B Detection Antibody	1 bottle	12 mL	79799
Human Granzyme B Standard	1 vial	lyophilized	79801
Avidin-HRP E	1 bottle	12 mL	79627
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 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

# LEGEND MAX™ Human Granzyme B ELISA Kit

## 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 E	
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](http://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.

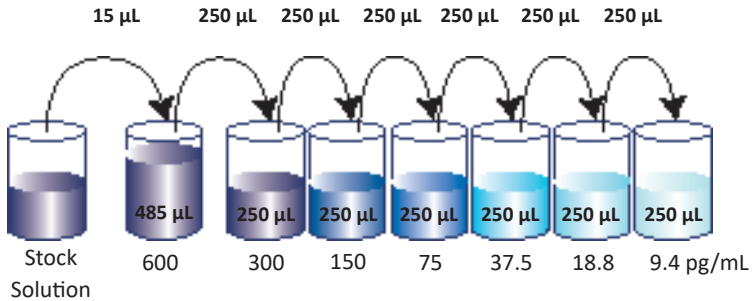
1. Dilute the 20X Wash Buffer to 1X with deionized water. For example, make 1 liter of 1X Wash Buffer by adding 50 mL of 20X Wash Buffer to 950 mL of deionized water. If crystals have formed in the 20X Wash Buffer, bring to room temperature and vortex until dissolved.
2. Reconstitute the lyophilized Human Granzyme B 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 10 minutes, then briefly vortex to mix completely.
3. In general, serum and plasma samples are assayed without dilutions. However, if dilutions are necessary, sample should be diluted with Assay Buffer A.
4. For cell culture supernatants samples, the end user may need to determine the dilution factors in a preliminary experiment. Samples should be diluted in Assay Buffer A if dilutions are necessary.

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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  $\mu\text{L}$  of the 600  $\text{pg}/\text{mL}$  top standard by diluting 15  $\mu\text{L}$  of the standard stock solution in 485  $\mu\text{L}$  of Assay Buffer A. Perform six two-fold serial dilutions of the 600  $\text{pg}/\text{mL}$  top standard in separate tubes using Assay Buffer A as the diluent. Thus, the human Granzyme B standard concentrations in the tubes are 600  $\text{pg}/\text{mL}$ , 300  $\text{pg}/\text{mL}$ , 150  $\text{pg}/\text{mL}$ , 75  $\text{pg}/\text{mL}$ , 37.5  $\text{pg}/\text{mL}$ , 18.8  $\text{pg}/\text{mL}$  and 9.4  $\text{pg}/\text{mL}$ , respectively. Assay Buffer A serves as the zero standard (0  $\text{pg}/\text{mL}$ ).



4. Wash the plate 4 times with at least 300  $\mu\text{L}$  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  $\mu\text{L}$  of Assay Buffer A to each well that will contain either standard dilutions or samples.
6. Add 50  $\mu\text{L}$  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.

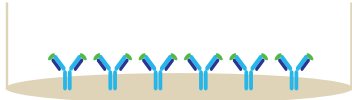


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8. Discard the contents of the plate into a sink, then wash the plate 4 times with 1X Wash Buffer as in step 4.
9. Add 100  $\mu$ L of Human Granzyme B Detection Antibody solution to each well, seal the plate and incubate at room temperature for 1 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  $\mu$ L of Avidin-HRP E 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 1 minute for each wash. This will help minimize background.
13. Add 100  $\mu$ L of Substrate Solution F to each well and incubate for 30 minutes in the dark. Wells containing human Granzyme B 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  $\mu$ L 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  $\mu$ L Assay Buffer A



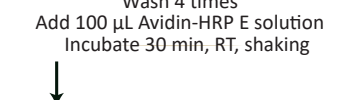
A diagram of a microplate well showing a surface with immobilized antibodies. Each antibody is represented as a blue Y-shape with green tips. The well is filled with a light brown liquid.
2. Add 50  $\mu$ L diluted standards or samples  
Incubate 2 hr, RT, shaking



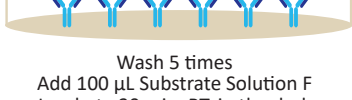
A diagram of a microplate well showing the same surface as in step 1. Purple Y-shaped molecules (representing standards or samples) are now bound to the green tips of the antibodies. The well is filled with a light brown liquid.
3. Wash 4 times  
Add 100  $\mu$ L Detection Antibody solution  
Incubate 1 hr, RT, shaking



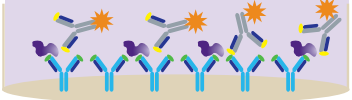
A diagram of a microplate well showing the same surface. In addition to the purple sample molecules, grey Y-shaped molecules (representing detection antibodies) are now bound to the green tips of the antibodies. The well is filled with a light brown liquid.
4. Wash 4 times  
Add 100  $\mu$ L Avidin-HRP E solution  
Incubate 30 min, RT, shaking



A diagram of a microplate well showing the same surface. In addition to the grey detection antibodies, orange star-shaped molecules (representing Avidin-HRP) are now bound to the green tips of the antibodies. The well is filled with a light brown liquid.
5. Wash 5 times  
Add 100  $\mu$ L Substrate Solution F  
Incubate 30 min, RT, in the dark



A diagram of a microplate well showing the same surface. The well is now filled with a light purple liquid, indicating color development. The orange star-shaped molecules are still bound to the antibodies.
6. Add 100  $\mu$ L Stop Solution



A diagram of a microplate well showing the same surface. The well is now filled with a light yellow liquid, indicating that the color development has been stopped. The orange star-shaped molecules are still bound to the antibodies.
7. Read absorbance at 450 nm and 570 nm

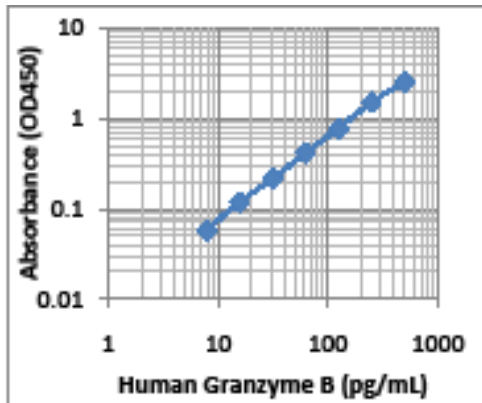
## Calculation of Results:

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.



## Performance Characteristics:

**Specificity:** No cross-reactivity was observed when this kit was used to analyze 32 human recombinant proteins listed below, each at 50 ng/mL.

Human	Granzyme A, Perforin, $\beta_2$ -Microglobulin, CD40L, Cystatin C, Eotaxin, GADPH, G-CSF, GM-CSF, ICAM-1, IP-10, Lipocalin-2, MCP-1, RANTES, sFasL, TIM-1, VCAM-1, CX3CL1, PDGF-BB, TGF- $\alpha$ , Lymphactin, GRO $\alpha$ , TNF- $\alpha$ , IL-34, IL-10, IL-8, IL-7, IL-6, IL-5, IL-4, IL-2, IL-1 $\beta$
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**Sensitivity:** The minimum detectable concentration is  $2.4 \pm 1.2$  pg/mL (n = 5).

**Recovery:** Recombinant human Granzyme B, at concentrations of 300, 75, and 18.8 pg/mL, was spiked into 6 human serum samples and then analyzed with the LEGEND MAX™ Human Granzyme B ELISA Kit. On average, 93.9% of the protein was recovered from the serum samples.

**Linearity:** Six human serum samples were spiked with recombinant human Granzyme B and serially diluted with Assay Buffer A to produce samples with values within the dynamic range and then assayed with the kit. The linearity of dilution ranged from 104.4 to 112.8%. On average, 109.2% linearity of dilution was observed.

**Intra-Assay Precision:** Two samples with different Granzyme B concentrations were tested with 16 replicates in one assay.

Concentration	Sample 1	Sample 2
Number of Replicates	16	16
Mean Concentration (pg/mL)	255.8	95.7
Standard Deviation	16.9	2.5
% CV	7%	3%

**Inter-Assay Precision:** Two samples with different concentrations of Granzyme B were assayed in six independent assays.

Concentration	Sample 1	Sample 2
Number of Assays	6	6
Mean Concentration (pg/mL)	270.1	91.4
Standard Deviation	13.8	9.3
% CV	5%	10%

### **Biological Samples:**

***Serum and plasma*** - Normal human serum (n = 22) and plasma (n = 16) samples were assayed for basal levels of human Granzyme B. Sample levels ranged from non-detectable to 9.0 pg/mL, averaging 1.0 pg/mL.

***Cell Culture Supernatant*** - Human PBMCs were plated at  $1 \times 10^6$  /ml in complete RPMI medium and stimulated under various conditions. Supernatants were harvested on day 2 and assayed for human Granzyme B. The results are summarized in the table below:

Stimulation Conditions	Conc. (pg/mL)
Unstimulated Control	92.4
CD3 plus CD28 (1 µg/mL each)	88,300
LPS (100 ng/mL)	68.4

**Troubleshooting Guide:**

<b>Problem</b>	<b>Probable Cause</b>	<b>Solution</b>
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 <sub>3</sub> )	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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