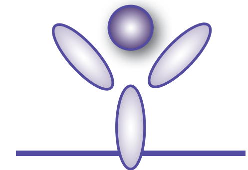
LEGEND MAX™

ELISA Kit with Pre-coated Plates



High Sensitivity Human IFN-γ

Cat. No. 430117

ELISA Kit for Accurate Quantitation of Human IFN-γ from Serum, Heparin Plasma, and Cell Culture Supernatant

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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Introduction:

Human IFN-γ (Immune interferon, Type II interferon, T cell interferon, Macrophage-activating factor (MAF), IFN-γ, IFN-gamma, etc.) is a potent multifunctional cytokine that is secreted primarily by activated NK cells and T cells. Originally characterized based on its anti-viral activities, IFN-γ also exerts anti-proliferative, immunoregulatory, and proinflammatory activities. IFN-γ can upregulate MHC class I and II antigen expression by antigen-presenting cells. Natural IFN-γ is a dimeric protein with subunits of 143 amino acids. IFN-γ is synthesized as a precursor protein of 166 amino acids, including a secretory signal sequence of 23 amino acids. The protein is glycosylated.

The BioLegend LEGEND MAX™ High Sensitivity Human IFN-γ 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 IFN-γ antibody. The detection antibody is a biotinylated mouse monoclonal anti-human IFN-γ antibody. After washing away any unbound biotinylated detection antibody, a streptavidin-polymer HRP is used for detection. Streptavidin-Polymer HRP enhances the sensitivity of the kit, allowing for detection of low concentrations of IFN-γ. This kit is specifically designed for the accurate quantitation of human IFN-γ from serum, heparin plasma, and cell culture supernatant. This kit is analytically validated with ready-to-use reagents.

Materials Provided:

Description	Quantity	Volume (per bottle)	Part #
Anti-human IFN-γ Pre-coated 96-well Strip Microplate	1 plate		750002751
Human IFN-γ Detection Antibody	1 bottle	12 mL	750004009
Human IFN-γ Lyophilized Standard	1 vial	lyophilized	750004011
Streptavidin-Polymer HRP	1 bottle	12 mL	750002513
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	1 pack		78101

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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.
Streptavidin-Polymer HRP	
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.

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.

Heparin Plasma: Collect blood samples in heparin-containing tubes. 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.

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.

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 IFN-γ Standard by adding the volume of Assay Buffer A to make the standard stock solution (refer to LEGEND MAX™ Kit Lot-Specific Certificate of Analysis). Allow the reconstituted standard to sit at room temperature for 15-20 minutes, then briefly vortex to mix completely.
3. In general, serum and heparin plasma samples are analyzed without dilution. The cell culture supernatant dilution factor should be determined by the end-user. If dilutions are required, use Assay Buffer A as the diluent.

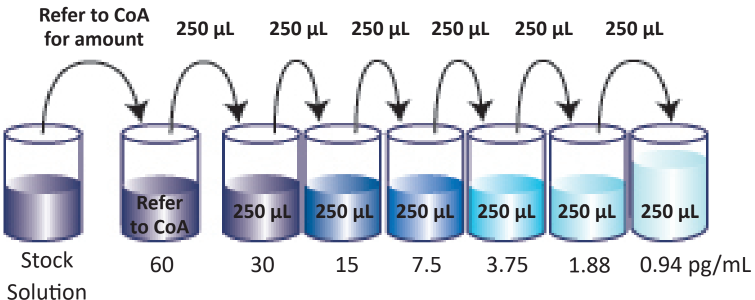
Assay Procedure:

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 60 pg/mL top standard by diluting the appropriate amount of the standard stock solution in Assay Buffer A (refer to the LEGEND MAX™ Kit Lot-Specific Certificate of Analysis). Perform six two-fold serial dilutions of the 60 pg/mL top standard in separate tubes using Assay Buffer A as the diluent. Thus, the human IFN-γ standard concentrations in the tubes are 60 pg/mL, 30 pg/mL, 15 pg/mL, 7.5 pg/mL, 3.75 pg/mL, 1.88 pg/mL and 0.94 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 the plate upside down on absorbent paper. All subsequent washes should be performed similarly.

5. Add 50 µL of Assay Buffer A 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 with shaking.

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 Human IFN-γ Detection Antibody to each well, seal the plate

and incubate at room temperature for 1 hour with 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 Streptavidin-Polymer HRP to each well, seal the plate, and incubate at room temperature for 30 minutes with 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 F to each well and incubate for 15 minutes* **in the dark**. Wells containing human IFN-γ 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 within 15 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.

*** Optimal substrate incubation time depends on laboratory conditions and the optical linear ranges of ELISA plate readers.**

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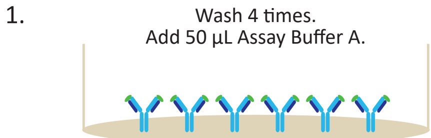
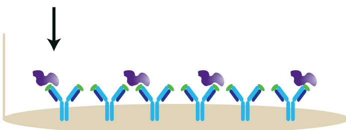
Assay Procedure Summary

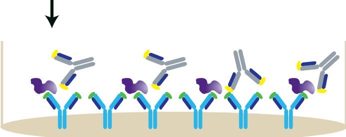
1. Wash 4 times.
   Add 50 µL Assay Buffer A.



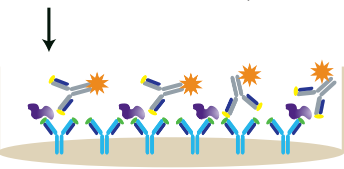
2. Add 50 µL diluted standards or samples.
   Incubate 2 hr, RT on plate shaker.



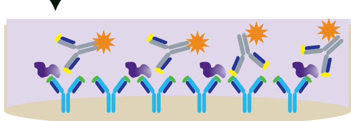
3. Wash 4 times.
   Add 100 µL Detection Antibody.
   Incubate 1 hr, RT on plate shaker.



4. Wash 4 times.
   Add 100 µL Streptavidin-Polymer HRP.
   Incubate 30 min, RT on plate shaker.



5. Wash 5 times.
   Add 100 µL Substrate Solution F.
   Incubate 15 min*, RT on benchtop, **in the dark.**



6. Add 100 µL Stop Solution.

7. Read absorbance at 450 nm and 570 nm.

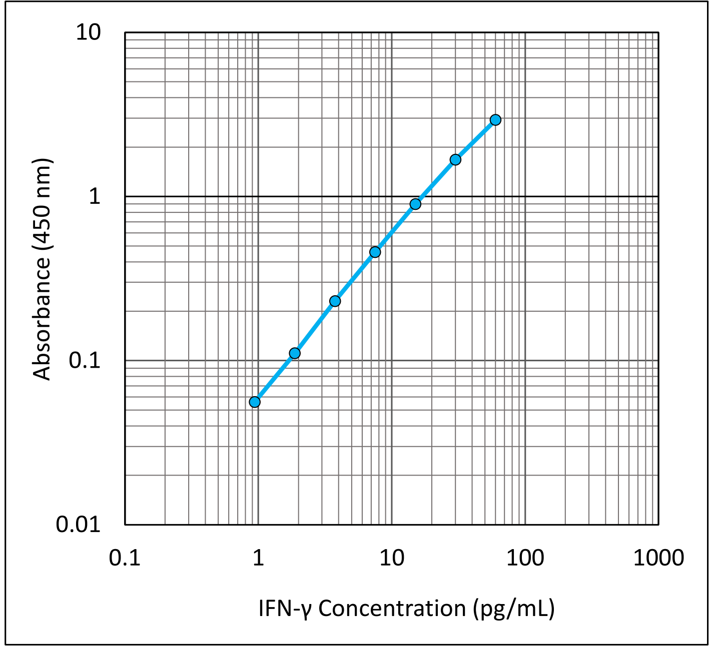
Calculation of Results:

The data can be best calculated with computer-based curve-fitting software using a 5- or 4-parameter logistics curve-fitting algorithm. If the 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 analyte concentration on the X-axis and absorbance on the Y-axis. Draw a best fit line through the standard points. To determine the unknown analyte 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 analyte 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 multiple human proteins (IFN-α2, IFN-β, IL-28A, IFN-λ1, IFN-γ R1), mouse IFN-γ, and rat IFN-γ at 50 ng/mL.

Sensitivity: The minimum detectable concentration of human IFN-γ is 0.267 ± 0.054 pg/mL.

Recovery: Recombinant human IFN-γ was spiked into two human serum samples and into cell culture supernatant (from unstimulated PBMCs) at three different concentrations. Then, sample recovery was analyzed with the LEGEND MAX™ High Sensitivity Human IFN-γ ELISA kit. On average, 77.7 % of the cytokine was recovered from the serum samples; 103.3% was recovered from the cell culture supernatant.

Linearity: Serum and heparin plasma samples were spiked with a high concentration of recombinant human IFN-γ. Cell culture supernatant (from PBMCs stimulated with 10 µg/mL CD3 and 1 µg/mL CD28 for 2 days) was not spiked. The samples were serially diluted two-fold to produce samples with concentrations within the dynamic range. Then, linearity was analyzed with the LEGEND MAX™ High Sensitivity Human IFN-γ ELISA kit.

Sample Type	N	Linearity (%)
Serum	4	95.8%
Heparin Plasma	4	102.7%
Cell Culture Supernatant (CD3/CD28-stimulated PBMCs)	1	99.7%

Intra-Assay Precision: Sixteen replicates of each of two samples containing different human IFN-γ concentrations were tested in one assay.

Concentration	Sample 1	Sample 2
Number of Replicates	16	16
Mean Concentration (pg/mL)	8.19	1.15
Standard Deviation	0.24	0.087
%CV	2.97	7.55

Inter-Assay Precision: Two samples containing different concentrations of human IFN-γ were tested in ten independent assays.

Concentration	Sample 1	Sample 2
Number of Assays	10	10
Mean Concentration (pg/mL)	7.51	1.21
Standard Deviation	0.88	0.11
%CV	11.76	8.84

Biological Samples: Freshly isolated human peripheral blood mononuclear cells (PBMCs) at a concentration of 1 x 10^6 cells/mL were stimulated with 10 µg/mL of plate-coated anti-CD3 antibody and 1 µg/ml of soluble anti-CD28 antibody at 37°C for two days. Cell culture supernatant was collected and assayed for natural human IFN-γ. The resulting human IFN-γ concentration was 10,205 pg/mL in anti-CD3 and anti-CD28 double-stimulated cell supernatant. IFN-γ was undetectable in the unstimulated samples.

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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												

Notes

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