Cat. # MK153

For Research Use

TaKaRa

Human IFN gamma EIA Kit

Product Manual

v201607

Table of Contents

I. Description

Interferon gamma (IFN gamma, IFN- g) is a cytokine that is primarily produced by T cells, NK cells, and $CD4^+/CD8^+$ lymphocytes when activated by antigens, mitogens, and allogeneic antigens. IFN gamma inhibits the IL-4-induced proliferation of B cells. It is also known to inhibit the growth of smooth muscle cells of the arterial intima *in vitro* and *in vivo*, and is hypothesized to inhibit excessive vascular expansion in response to arterial damage caused by stenosis.
Human IFN gamma is a 143 amino acid protein that is glycosylated in two locations. The biologically active form of IFN gamma exists as a dimer.

The Human IFN gamma EIA Kit is a sandwich-type EIA that uses two monoclonal antibodies to assay human IFN gamma in serum, plasma, and cell culture supernatant. The kit includes an assay plate that is precoated with antibody, allowing for a simple protocol and high reproducibility. This EIA kit is based on a avidin/biotin system and can be used for high-sensitivity measurement of IFN gamma in multiple samples in -3 hours.

II. Principle

The kit includes a 96-well plate that has been coated with antibodies that are specific to human IFN gamma. To begin the assay, the standard solution and unknown samples are added to each well. The immobilized antibodies on the plate capture the human IFN gamma in the sample during incubation. Next, after washing, a biotin-labeled anti-human IFN gamma antibody is added. After incubation, the plate is washed to remove free antibody, and HRP-labeled streptavidin is added to the wells. After TMBZ substrate solution is added, a blue color develops; the intensity of the color is directly proportional to the concentration of bound IFN gamma. When the reaction stop solution is added, the color changes from blue to yellow. Absorbance at 450 nm is measured using a plate reader, and analysis is carried out using the value obtained.

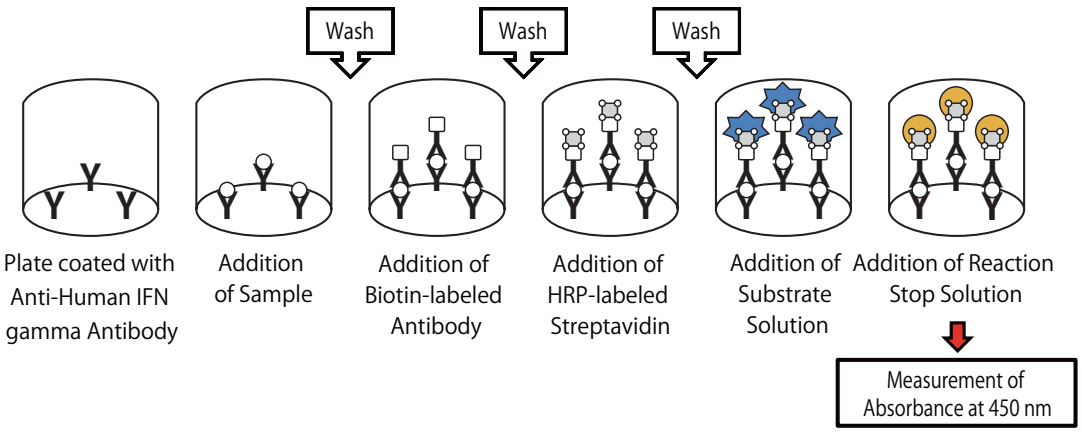


Figure 1. Overview of Human IFN gamma EIA Kit procedure.

III. Components

(1) Antibody Coated Microtiterplate — 1 plate
Anti-Human IFN gamma antibody-coated plate
(96 wells: 8 wells x 12 strips)

(2) Wash Buffer Concentrate(20X) — 25 ml
20-times concentrated wash solution

(3) Standard human IFN (lyophilized) — 2 vials, for 1 ml
Recombinant human IFN gamma (from *E. coli*) 20 ng

(4) Assay Diluent A — 30 ml
Sample diluent for serum or plasma
* Includes 0.09% sodium azide as a preservative

(5) Assay Diluent B (5X) — 15 ml
Diluent used to prepare samples of cell culture supernatant and reagents

(6) Detection Antibody human IFN (lyophilized) — 2 vials, for 0.1 ml
Biotin-labeled human IFN gamma antibody

(7) HRP-Streptavidin Concentrate — 0.2 ml
HRP-labeled streptavidin concentrate

(8) Substrate Solution(TMBZ) — 12 ml
3,3' ,5,5' - Tetramethylbenzidine solution

(9) Stop Solution without Sulfuric Acid — 12 ml
Reaction stop solution (with no sulfuric acid)

IV. Materials Required but not Provided

- Microplate reader (that can measure absorbance at 450 nm)
- Micropipette and tips
- Multi-channel pipette
- Plate-washing machine

  Note 1 : A Personal Microplate Washer (Cat. #MK950)＊ can be used.
  ＊ : Not available in all geographic locations. Check for availability in your area.

  Note 2 : If there is insufficient wash buffer for use in an automated ELISA processing system, prepare 0.1% Tween 20/PBS using "Wash and Stop Solution for ELISA without Sulfuric Acid" (Cat. #MK021) and use as the wash buffer.
- Plate mixer
- Incubator (20 - 30℃)
- Pipettes for 1 - 25 ml (for reagent preparation)
- 100 ml and 1 L graduated cylinders
- Paper towels
- Distilled water
- Software to analyze ELISA data
- Tubes for specimen dilution and preparation of standard solution

V. Storage

- Store unopened kit at -20℃. Avoid repeated freezing and thawing. Once thawed, stored at 2 - 8℃ for -6 months.
- After opening, the microplate and reagents can be stored for -1 month at 2 - 8℃. Store any unused microplate wells in the provided pouch with desiccant.

VI. Purpose of Use

Measurement of human IFN gamma in serum, plasma, or cell culture supernatant.

VII. Protocol

Bring all reagents and samples to room temperature (20 - 30℃) before use.

1. Preparation of Reagents

- Bring the antibody plate [(1) Antibody Coated Microtiterplate] to room temperature before use and unseal.

- Wash solution
  Dilute 25 ml of (2) Wash Buffer Concentrate (20X) with 475 ml of distilled water and mix well.
  * If a precipitate is observed, heat to room temperature and mix gently to dissolve.
  * When an automated ELISA processing system is used, there may be insufficient wash buffer. In this case, prepare 0.1% Tween 20/PBS using Wash and Stop Solution for ELISA without Sulfuric Acid (Cat. #MK021).

- Assay Diluent A (for dilution of plasma and serum samples)
  Use (4) Assay Diluent A directly. It contains 0.09% sodium azide as a preservative. When measuring IFN gamma in plasma or serum samples, use to dilute the standard solution and the samples.

- Assay Diluent B (for dilution of cell culture supernatant and reagent preparation)
  Prepare 1X Assay Diluent B by diluting (5) Assay Diluent B (5X) 5 times with distilled water. When measuring IFN gamma in cell culture media or supernatant, use for dilution of the standard solution and the samples.

- Human IFN gamma Standard Solution
  Spin down the vial of (3) Standard Human IFN briefly before opening. Add 1 ml of Assay Diluent A or 1X Assay Diluent B to the vial (use the same diluent for sample dilution) and mix carefully until the powder is completely dissolved to prepare 20 ng/ml human IFN gamma standard solution. The reconstituted human IFN gamma standard solution can be stored at -20℃ or below (-80℃ is recommended).
  Prepare serial dilutions of the standard solution following the procedures in step VII-3.

- Biotin-labeled anti-human IFN gamma antibody
  Spin down the vial of (6) Detection Antibody Human IFN briefly before opening. Add 100 μl of 1X Assay Diluent B to the vial and mix gently by pipetting to prepare a stock solution of biotin-labeled anti-human IFN gamma antibody. After preparation, the solution can be stored for 5 days at 4℃.
  At the time of use, dilute 80-fold with 1X Assay Diluent B.
  One vial contains enough for the measurement of 48 wells (two vials are included in this kit).

- HRP-labeled streptavidin [(7) HRP-Streptavidin Concentrate]
  Spin down the vial of (7) HRP-Streptavidin Concentrate before opening.
  Dilute 200 - fold with 1X Assay Diluent B for use.

  Preparation example:

  Spin down and open.
  Carefully mix by pipetting.
  Remove 60 μl (amount adequate for 1 plate) and add 12 ml of 1X Assay Diluent B.
  Use the prepared HRP-labeled streptavidin within 24 hours. The diluted solution cannot be stored.

- Reaction stop solution [(9) Stop Solution without Sulfuric Acid]
  Use directly.
  * Because this is a highly viscous solution, agitate well with a plate mixer after addition. The color reaction is stable for 6 hours.

2. Samples

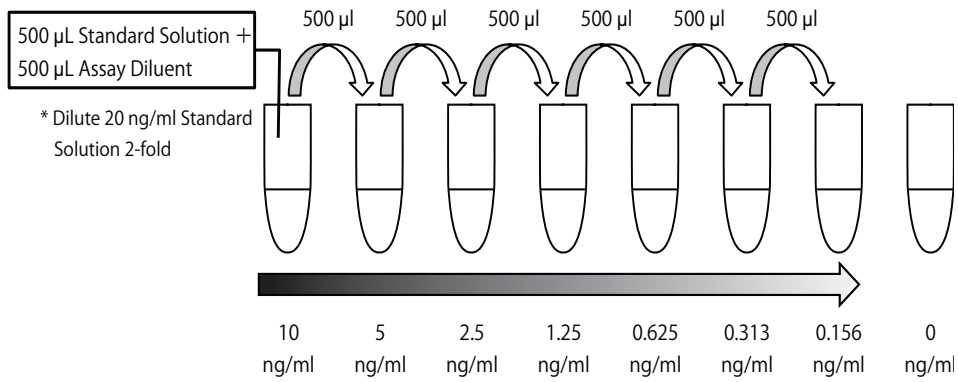
- Use (4) Assay Diluent A to dilute plasma and serum samples.
- Use 1X Assay Diluent B to dilute cell culture supernatant samples.
- For standard solutions, measurement accuracy can be improved by using the same diluent as used for sample dilution.

Standard dilution for normal serum and plasma: >2 - fold
Standard dilution for culture supernatant of stimulated lymphocytes: 2 - 8-fold

* IFN gamma expression differs depending on the sample; determine the optimal dilution experimentally.

3. Preparation of standard dilution for a standard curve

Using the same diluent that was used to prepare the human IFN gamma standard solution (20 ng/ml), prepare a 2-fold dilution series of the 20 ng/ml stock solution (10, 5, 2.5, 1.25, 0.625, 0.313, and 0.156 ng/ml). Mix well at each dilution step. Changing the pipette tip between dilutions will improve accuracy. Also prepare a 0 ng/ml standard using diluent only (Assay Diluent A or 1X Assay Diluent B).



Repeat 2-fold serial dilutions by adding 500 µl of standard solution to 500 µL of Assay Diluent.

Figure 2. Preparing the standard solutions.

4. Procedure

Bring reagents and samples to room temperature (20 - 30℃) before use. Measure standard solutions and samples in at least duplicate.

1. Add 100 μl of each concentration of the standard solution and the samples to each well. Cover the wells and allow to incubate at room temperature for 1 hour.
   * If IFN gamma content of the sample is low or if there are a large number of samples, the reaction can be performed overnight at 4℃.

2. Remove the reaction solution and wash 4 times with 300 μl wash solution. Use a plate-washing machine or a multi-channel pipette to add the wash solution to each well. It is important to remove reaction mixture completely at each step. After washing, remove the wash solution well by aspiration (suction) or by decanting. Further, turn the plate upside down and then hit it against a paper towel on a hard surface several times to drain the liquid.

3. Add 100 μl of the prepared biotin-labeled antibody to each well. Incubate at room temperature for 1 hour.

4. Remove the reaction solution and wash 4 times with the wash solution following the procedures in step 2.

5. Add 100 μl of the prepared HRP-labeled streptavidin to each well. Incubate at room temperature for 30 minutes.

6. Remove the reaction solution and wash 4 times with the wash solution following the procedures in step 2.

7. Add 100 μl of Substrate Solution (TMBZ) to each well. Allow the color reaction progress for 15 minutes at room temperature in the dark.

8. Add 100 μl of Reaction Stop Solution to each well in the same order that the Substrate Solution was added, and mix well using a plate mixer.

9. Measure absorbance at 450 nm using a plate reader.

(Summary of measurement method)

1. Prepare the reagents.
   Prepare samples and standard solutions.

⇩

2. Add 100 μl of each sample and standard solution to each well.
   Incubate for 1 hour at room temperature or overnight at 4℃.
   First reaction

⇩ Wash

3. Add 100 μl of biotin-labeled antibody to each well.
   Incubate at room temperature for 1 hour.
   Second reaction

⇩ Wash

4. Add 100 μl of HRP-labeled streptavidin to each well.
   Incubate at room temperature for 30 minutes.
   Third reaction

⇩ Wash

5. Add 100 μl of TMBZ to each well.
   Incubate at room temperature for 15 minutes.

⇩

6. Add 100 μl of Stop solution to each well.
   Measure absorbance at 450 nm.

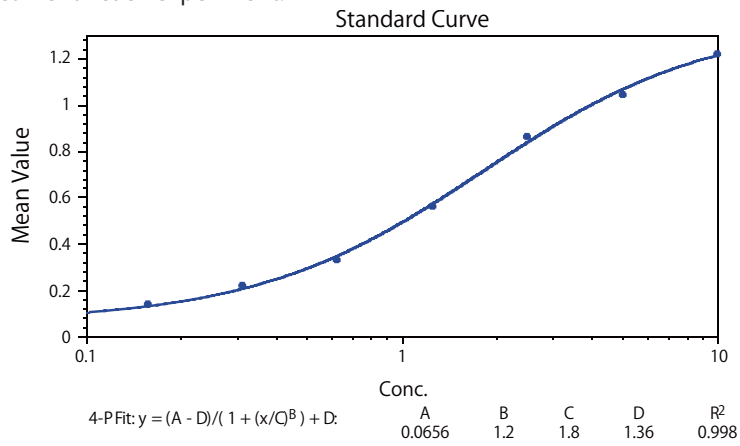
5. Calculation

A standard curve is prepared by plotting the results of standard solutions from duplicate measurements, with concentration as the x-axis and absorbance as the y-axis. Based on the absorbance value of the standards and sample, the sample concentration is calculated from the standard curve.
If the absorbance of the 0 ng/ml standard is greater than 0.2, perform background subtraction by subtracting the absorbance of the zero concentration from all the other measured values.

VIII. Performance

1. Standard Curve

The following standard curve is a representative example. Prepare a standard curve for each experiment.

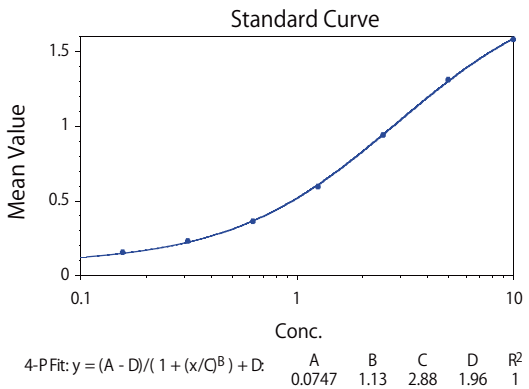


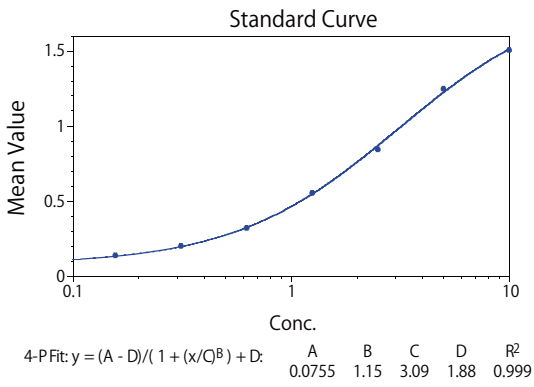
Human IFN gamma (ng/ml)	10	5	2.5	1.25	0.625	0.313	0.156	0
A_{450}	1.220	1.044	0.866	0.562	0.331	0.219	0.139	0.057

<Standard Curve Comparison using different diluents>

Assay Dilution A		
Sample	Concentration (ng/ml)	A_{450}
Std 01	10.0	1.581
Std 02	5.0	1.310
Std 03	2.5	0.941
Std 04	1.25	0.593
Std 05	0.625	0.362
Std 06	0.313	0.224
Std 07	0.156	0.152
Std 08	0.0	0.065

1 X Assay Dilution B		
Sample	Concentration (ng/ml)	A_{450}
Std 09	10.0	1.503
Std 10	5.0	1.246
Std 11	2.5	0.846
Std 12	1.25	0.555
Std 13	0.625	0.319
Std 14	0.313	0.202
Std 15	0.156	0.139
Std 16	0.0	0.067





2. Sensitivity

Minimum sensitivity of detection of human IFN gamma: 78 pg/ml

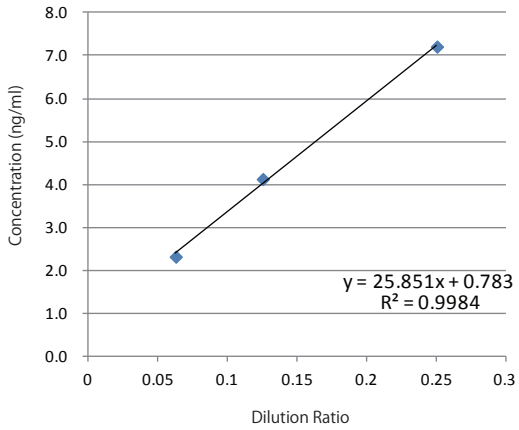
3. Spike and Recovery Testing

Spike and recovery testing was performed by spiking human serum, plasma, and cell culture supernatant with known quantities of human IFN gamma.

Specimen	Mean Recovery (%)	Range (%)
Serum	88.65	82 - 103
Plasma	86.82	81 - 102
Cell Culture Supernatant	94.53	84 - 104

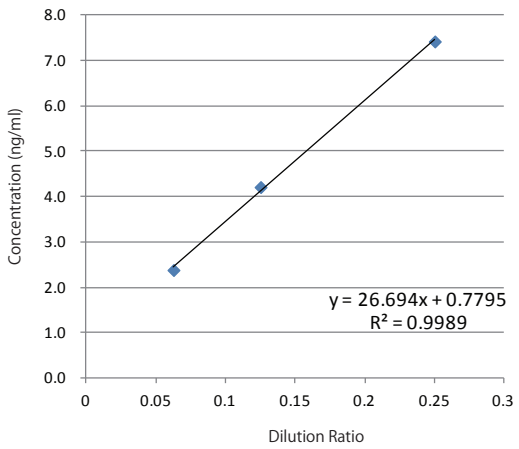
4. Linearity of Dilution

<Dilution of 100% fetal bovine serum spiked with a human IFN gamma (Diluent A)>



Dilution		Concentration (ng/ml)
2X	(0.5)	9.654
4X	(0.25)	7.208
8X	(0.125)	4.128
16X	(0.063)	2.323

<Dilution of RPM1640 culture medium with 10% bovine calf serum spiked with human IFN gamma (Diluent B)>



Dilution		Concentration (ng/ml)
2X	(0.5)	7.955
4X	(0.25)	7.421
8X	(0.125)	4.212
16X	(0.063)	2.384

Caution: When the concentrations are near the highest concentrations of the standard curve, dilution linearity decreases.

5. Reproducibility

Intra-day (repeatability) : CV < 10%
Inter-day (reproducibility) : CV < 12%

6. Specificity

No cross-reactivity with the following cytokines:

Human Angiogenin		BDNF	BLC	ENA-78	FGF-4
IL-1 α	IL-1 β	IL-2	IL-3	IL-4	IL-5
IL-7	IL-8	IL-9	IL-10	IL-11	IL-12p70
IL-12p40	IL-13	IL-15	IL-309	IP-10	G-CSF
GM-CSF	MCP-1	MCP-2	MCP-3	MDC	MIP-1 α
MIP-1 β	MIP-1 σ	PARC	PDGF	RANTES	SCF
TARC	TGF- β	TIMP-1	TIMP-2	TNF- α	TNF- β
TPO	VEGF				

7. Information for use

- No cross-reactivity has been observed with bovine antigens, therefore measurement is not affected by FCS (fetal calf serum) in the culture media.
- When measuring IFN gamma produced from cells such as human PBMC (peripheral blood mononuclear cells), NK cells, and T cells cultured in culture media that contains human AB blood serum or autologous blood serum, perform a control experiment using only culture media only to measure the basal level of IFN gamma.
- When measuring IFN gamma in human serum or plasma, start with a 2 - fold dilution. With samples from subjects that have received antigen vaccines, that have inflammation, or that have a history of viral infection, preliminary investigation of dilution may be necessary as there is a possibility of high IFN gamma values in these samples.
- For measurement samples of culture supernatant of PBMC, NK cells, or T cells that have been stimulated with CD3 antibody, PMA (phorbol ester), or antigen, the concentration of IFN gamma may increase over time. Select a dilution ratio that accounts for this.
  When using PBMC, the response is less dramatic in comparison with NK cells and cultured T cells, even when the purity of the cell population is taken into consideration.

<Cytokine Induction Conditions for Cultured T Cells using CD3 Antibody Stimulation>

CD3 antibody stimulation conditions:
Antibody Concentration: 1 μg/ml, 0.1 ml/well of a 96 well plate

Cultured T cells:
1 - 2 x 10^5 cells in 0.2 ml/well, 96 well plate

Time points:
24 hours, 48 hours, 72 hours, and 5 days

Dilution of samples:
Measurement using a 3-step dilution (2X, 4X, and 8X) at each timepoint.

IX. References

1) De Maeyer. E and De Maeyer-Guignard, J. (1992) Interferon-gamma. *Current Opinion in Immunology.* **4**:321-326.
2) Dayton, M.A. *et al*. (1992) Human B cell lines express the interferon gamma gene. *Cytokine.* **4**:454-460.
3) Gu, D. and Sarvetnick, N. (1993) Epithelial cell proliferation and islet neogenesis in IFN-gamma transgenic mice. *Development.* **118**:33-46.

X. Related Products

Mouse IFN gamma EIA Kit (Cat. #MK152)
Pig IFN gamma EIA Kit (Cat. #MK154)