ab236895
Human IFN gamma
High Sensitivity
SimpleStep ELISA® Kit

For the quantitative measurement of IFN gamma in human serum, plasma, saliva, and cell culture supernatants.

This product is for research use only and is not intended for diagnostic use.

Table of Contents

1. Overview

IFN gamma High Sensitivity *in vitro* SimpleStep ELISA® (Enzyme-Linked Immunosorbent Assay) kit is designed for the quantitative measurement of IFN gamma protein in human serum, plasma, saliva, and cell culture supernatants.

The SimpleStep ELISA® employs an affinity tag labeled capture antibody and a reporter conjugated detector antibody which immunocapture the sample analyte in solution. This entire complex (capture antibody/analyte/detector antibody) is in turn immobilized via immunoaffinity of an anti-tag antibody coating the well. To perform the assay, samples or standards are added to the wells, followed by the antibody mix. After incubation, the wells are washed to remove unbound material. TMB Development Solution is added and during incubation is catalyzed by HRP, generating blue coloration. This reaction is then stopped by addition of Stop Solution completing any color change from blue to yellow. Signal is generated proportionally to the amount of bound analyte and the intensity is measured at 450 nm. Optionally, instead of the endpoint reading, development of TMB can be recorded kinetically at 600 nm.

IFN gamma (Interferon-gamma), is an important immunoregulatory cytokine in addition of having antiviral activity. It is a potent activator of macrophages, it has antiproliferative effects on transformed cells and it can potentiate the antiviral and antitumor effects of the type I interferons. IFN gamma is produced by lymphocytes activated by specific antigens or mitogens.

2. Protocol Summary

Prepare all reagents, samples, and standards as instructed

⇩

Add 50 μL standard or sample to appropriate wells

⇩

Add 50 μL Antibody Cocktail to all wells

⇩

Incubate at room temperature for 1 hour

⇩

Aspirate and wash each well three times with 350 μL 1X Wash Buffer
PT

⇩

Add 100 μL TMB Development Solution to each well and incubate
for 10 minutes.

⇩

Add 100 μL Stop Solution and read OD at 450 nm

3. Precautions

Please read these instructions carefully prior to beginning the assay.

– All kit components have been formulated and quality control tested to function successfully as a kit.

– We understand that, occasionally, experimental protocols might need to be modified to meet unique experimental circumstances. However, we cannot guarantee the performance of the product outside the conditions detailed in this protocol booklet.

– Reagents should be treated as possible mutagens and should be handle with care and disposed of properly. Please review the Safety Datasheet (SDS) provided with the product for information on the specific components.

– Observe good laboratory practices. Gloves, lab coat, and protective eyewear should always be worn. Never pipet by mouth. Do not eat, drink or smoke in the laboratory areas.

– All biological materials should be treated as potentially hazardous and handled as such. They should be disposed of in accordance with established safety procedures.

4. Storage and Stability

Store kit at +4°C immediately upon receipt. Kit has a storage time of 1 year from receipt, providing components have not been reconstituted.

Refer to list of materials supplied for storage conditions of individual components.

5. Limitations

– Assay kit intended for research use only. Not for use in diagnostic procedures.
– Do not mix or substitute reagents or materials from other kit lots or vendors. Kits are QC tested as a set of components and performance cannot be guaranteed if utilized separately or substituted.

6. Materials Supplied

Item	Quantity	Storage Condition
Human IFN gamma High Sensitivity Capture Antibody 10X	600 µL	+4°C
Human IFN gamma High Sensitivity Detector Antibody 10X	600 µL	+4°C
Human IFN gamma High Sensitivity Lyophilized Recombinant Protein	2 Vials	+4°C
Antibody Diluent 4BI	6 mL	+4°C
Wash Buffer PT 10X	20 mL	+4°C
TMB Development Solution	12 mL	+4°C
Stop Solution	12 mL	+4°C
Sample Diluent NS	50 mL	+4°C
Sample Diluent 50BP	20 mL	+4°C
Anti-tag coated microplate (12 x 8 well strips)	96 Wells	+4°C
Plate Seal	1	+4°C

7. Materials Required, Not Supplied

These materials are not included in the kit, but will be required to successfully perform this assay:

– Microplate reader capable of measuring absorbance at 450 or 600 nm.
– Method for determining protein concentration (BCA assay recommended).
– Deionized water.
– Multi- and single-channel pipettes.
– Tubes for standard dilution.
– Plate shaker for all incubation steps.
– Optional: Phenylmethylsulfonyl Fluoride (PMSF) (or other protease inhibitors).

8. Technical Hints

– Samples generating values higher than the highest standard should be further diluted in the appropriate sample dilution buffers.
– Avoid foaming or bubbles when mixing or reconstituting components.
– Avoid cross contamination of samples or reagents by changing tips between sample, standard and reagent additions.
– Ensure plates are properly sealed or covered during incubation steps.
– Complete removal of all solutions and buffers during wash steps is necessary to minimize background.
– As a guide, typical ranges of sample concentration for commonly used sample types are shown below in Sample Preparation (section 11).
– All samples should be mixed thoroughly and gently.
– Avoid multiple freeze/thaw of samples.
– Incubate ELISA plates on a plate shaker during all incubation steps.
– When generating positive control samples, it is advisable to change pipette tips after each step.

- To avoid high background always add samples or standards to the well before the addition of the antibody cocktail.
- This kit is sold based on number of tests. A 'test' simply refers to a single assay well. The number of wells that contain sample, control or standard will vary by product. Review the protocol completely to confirm this kit meets your requirements. Please contact our Technical Support staff with any questions.

9. Reagent Preparation

–   Equilibrate all reagents to room temperature (18-25°C) prior to use. The kit contains enough reagents for 96 wells. The sample volumes below are sufficient for 48 wells (6 x 8-well strips); adjust volumes as needed for the number of strips in your experiment.

–   Sample Diluent BP may contain precipitate, this is normal. If precipitate is not dissolved by gentle mixing, the precipitate may be dissolved by gentle warming and mixing at 37°C for 10 minutes. If precipitate remains, gently spin down and avoid visible precipitates when pipetting.

–   Prepare only as much reagent as is needed on the day of the experiment. Capture and Detector Antibodies have only been tested for stability in the provided 10X formulations.

9.1   1X Wash Buffer PT:

Prepare 1X Wash Buffer PT by diluting Wash Buffer PT 10X with deionized water. To make 50 mL 1X Wash Buffer PT combine 5 mL Wash Buffer PT 10X with 45 mL deionized water. Mix thoroughly and gently.

9.2   Antibody Cocktail:

Prepare Antibody Cocktail by diluting the capture and detector antibodies in Antibody Diluent 4BI. To make 3 mL of the Antibody Cocktail combine 300 µL 10X Capture Antibody and 300 µL 10X Detector Antibody with 2.4 mL Antibody Diluent 4BI. Mix thoroughly and gently.
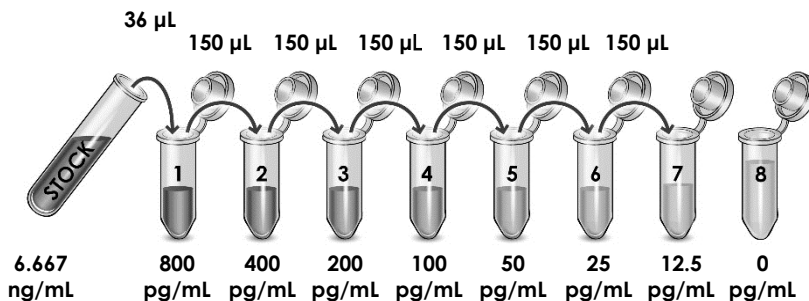
10. Standard Preparation

− Always prepare a fresh set of standards for every use.
− Discard working standard dilutions after use as they do not store well.
− The following section describes the preparation of a standard curve for duplicate measurements (recommended).

> **IMPORTANT:** If the protein standard vial has a volume identified on the label, reconstitute the IFN gamma by adding that volume of Diluent indicated on the label. Alternatively, if the vial has a mass identified, reconstitute the IFN gamma standard by adding 500 µL Diluent.

10.1 For **cell culture supernatant and saliva samples** follow these instructions**:**

10.1.1 Reconstitute the IFN gamma High Sensitivity standard sample by adding Sample Diluent NS. Hold at room temperature for 10 minutes and mix thoroughly and gently. This is the 6.667 ng/mL **Stock Standard** Solution.

10.1.2 Label eight tubes, Standards 1– 8.

10.1.3 Add 264 µL of Sample Diluent NS into tube number 1 and 150 µL of Sample Diluent NS into numbers 2-8.

10.1.4 Use the Stock Standard to prepare the following dilution series. Standard #8 contains no protein and is the Blank control:

36 µL							
	150 µL	150 µL	150 µL	150 µL	150 µL	150 µL	

STOCK	1	2	3	4	5	6	7	8
6.667 ng/mL	800 pg/mL	400 pg/mL	200 pg/mL	100 pg/mL	50 pg/mL	25 pg/mL	12.5 pg/mL	0 pg/mL

10.2 For **serum and plasma samples** follow these instructions:

10.2.1 Reconstitute the IFN gamma High Sensitivity standard sample by adding Sample Diluent 50BP. Hold at room temperature for 10 minutes and mix thoroughly and gently. This is the 6.667 ng/mL **Stock Standard** Solution.

10.2.2 Label eight tubes, Standards 1– 8.

10.2.3 Add 228 µL of Sample Diluent 50BP into tube number 1 and 150 µL of Sample Diluent 50BP into numbers 2-8.

10.2.4 Use the Stock Standard to prepare the following dilution series. Standard #8 contains no protein and is the Blank control:
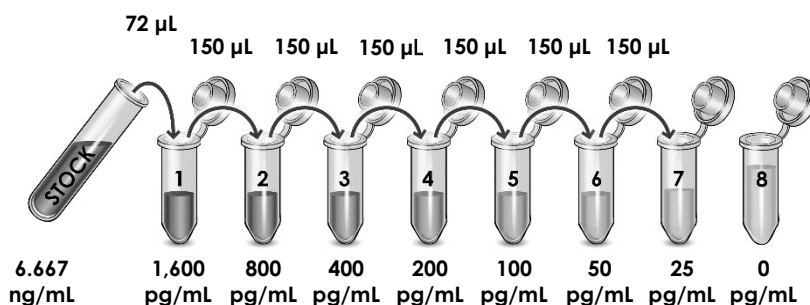
11. Sample Preparation

Typical Sample Dynamic Range	
Sample Type	Range
Serum*	≤100%
Plasma – Citrate*	≤100%
Plasma – EDTA*	≤100%
Saliva*	≤50%
PHA-M Stimulated PBMC cell culture supernatant	0.78 – 50%

*Based on spiked sample

11.1 Plasma:

Collect plasma using citrate or EDTA. Centrifuge samples at 2,000 x g for 10 minutes. Samples may be assayed neat or dilute samples into Sample Diluent 50BP and assay. Store un-diluted plasma samples at -20ºC or below for up to 3 months. Avoid repeated freeze-thaw cycles.

11.2 Serum:

Samples should be collected into a serum separator tube. After clot formation, centrifuge samples at 2,000 x g for 10 minutes and collect serum. Samples may be assayed neat or dilute samples into Sample Diluent 50BP and assay. Store un-diluted serum at -20ºC or below. Avoid repeated freeze-thaw cycles.

11.3 Cell Culture Supernatants:

Centrifuge cell culture media at 2,000 x g for 10 minutes to remove debris. Collect supernatants. Dilute samples at least 2-fold into Sample Diluent NS and assay. Store un-diluted samples at -20ºC or below. Avoid repeated freeze-thaw cycles.

11.4 Saliva:

Centrifuge saliva at 800 x g for 10 minutes to remove debris. Collect supernatants. Dilute samples at least 2-fold into Sample Diluent NS and assay. Store un-diluted samples at -20ºC or below. Avoid repeated freeze-thaw cycles.

12. Plate Preparation

− The 96 well plate strips included with this kit are supplied ready to use. It is not necessary to rinse the plate prior to adding reagents.

− Unused plate strips should be immediately returned to the foil pouch containing the desiccant pack, resealed and stored at 4°C.

− For each assay performed, a minimum of two wells must be used as the zero control.

− For statistical reasons, we recommend each sample should be assayed with a minimum of two replicates (duplicates).

− Differences in well absorbance or "edge effects" have not been observed with this assay.

13. Assay Procedure

- Equilibrate all materials and prepared reagents to room temperature prior to use.
- We recommend that you assay all standards, controls and samples in duplicate.

13.1 Remove excess microplate strips from the plate frame, return them to the foil pouch containing the desiccant pack, reseal and return to 4°C storage.

13.2 Remove excess microplate strips from the plate frame, return them to the foil pouch containing the desiccant pack, reseal and return to 4°C storage.

13.3 Add 50 µL of all sample or standard to appropriate wells.

13.4 Add 50 µL of the Antibody Cocktail to each well.

13.5 Seal the plate and incubate for 1 hour at room temperature on a plate shaker set to 400 rpm.

13.6 Wash each well with 3 x 350 µL 1X Wash Buffer PT. Wash by aspirating or decanting from wells then dispensing 350 µL 1X Wash Buffer PT into each well. Wash Buffer PT should remain in wells for at least 10 seconds. Complete removal of liquid at each step is essential for good performance. After the last wash invert the plate and tap gently against clean paper towels to remove excess liquid.

13.7 Add 100 µL of TMB Development Solution to each well and incubate for 10 minutes in the dark on a plate shaker set to 400 rpm.
Given variability in laboratory environmental conditions, optimal incubation time may vary between 5 and 20 minutes.
<u>*Note*</u>*: The addition of Stop Solution will change the color from blue to yellow and enhance the signal intensity about 3X. To avoid signal saturation, proceed to the next step before the high concentration of the standard reaches a blue color of O.D.600 equal to 1.0.*

13.8 Add 100 µL of Stop Solution to each well. Shake plate on a plate shaker for 1 minute to mix. Record the OD at 450 nm. This is an endpoint reading.

13.9 Alternative to 13.7 – 13.8: Instead of the endpoint reading at 450 nm, record the development of TMB Substrate kinetically. Immediately after addition of TMB Development Solution begin recording the blue color development with elapsed

time in the microplate reader prepared with the following settings:

Mode	Kinetic
Wavelength:	600 nm
Time:	up to 20 min
Interval:	20 sec - 1 min
Shaking:	Shake between readings

Δ **Note**: that an endpoint reading can also be recorded at the completion of the kinetic read by adding 100 μL Stop Solution to each well and recording the OD at 450 nm.

13.10 Analyze the data as described below.

14. Calculations

14.1 Calculate the average absorbance value for the blank control (zero) standards. Subtract the average blank control standard absorbance value from all other absorbance values.

14.2 **Create a standard curve** by plotting the average blank control subtracted absorbance value for each standard concentration (y-axis) against the target protein concentration (x-axis) of the standard. Use graphing software to draw the best smooth curve through these points to construct the standard curve.

Δ **Note:** Most microplate reader software or graphing software will plot these values and fit a curve to the data. A four-parameter curve fit (4PL) is often the best choice; however, other algorithms (e.g. linear, semi-log, log/log, 4-parameter logistic) can also be tested to determine if it provides a better curve fit to the standard values.

14.3 Determine the concentration of the target protein in the sample by interpolating the blank control subtracted **absorbance values against the standard curve**. Multiply the resulting value by the appropriate sample dilution factor, if used, to obtain the concentration of target protein in the sample.

14.4 Samples generating absorbance values greater than that of the highest standard should be further diluted and reanalyzed. Similarly, samples which measure at an absorbance values less than that of the lowest standard should be retested in a less dilute form.

15. Typical Data

Typical standard curve – data provided **for demonstration purposes only**. A new standard curve must be generated for each assay performed.
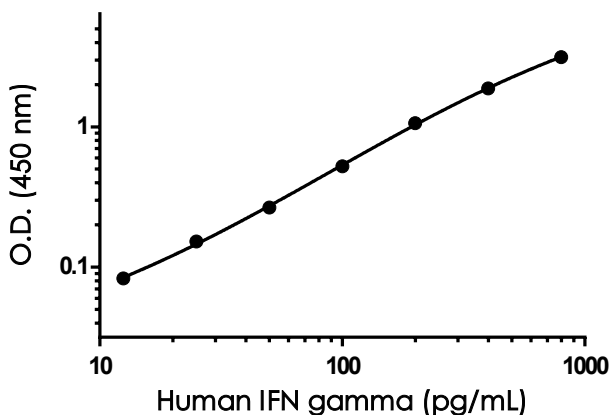
Standard Curve Measurements			
Concentration (pg/mL)	O.D 450 nm		Mean O.D
	1	2	
0	0.118	0.119	0.118
12.5	0.201	0.202	0.201
25	0.264	0.277	0.271
50	0.373	0.394	0.385
100	0.633	0.653	0.643
200	1.187	1.180	1.184
400	2.011	1.986	1.998
800	3.273	3.263	3.268

Figure 1. Example of human IFN gamma standard curve in Sample Diluent NS. The IFN gamma standard curve was prepared as described in Section 10. Raw data values are shown in the table. Background-subtracted data values (mean +/- SD) are graphed.
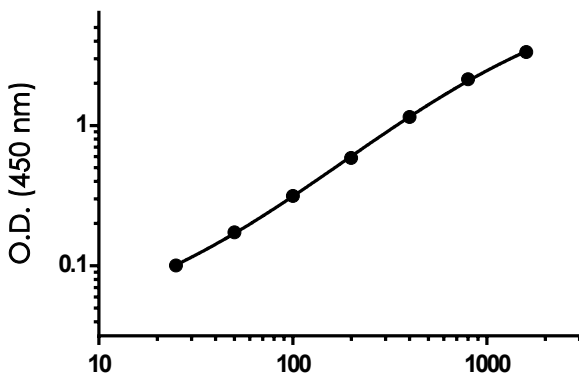
Standard Curve Measurements			
Concentration (pg/mL)	O.D 450 nm		Mean O.D
	1	2	
0	0.108	0.101	0.105
25	0.207	0.203	0.205
50	0.280	0.276	0.278
100	0.421	0.418	0.419
200	0.685	0.701	0.693
400	1.239	1.271	1.255
800	2.224	2.257	2.240
1,600	3.464	3.429	3.446

Figure 2. Example of human IFN gamma standard curve in Sample Diluent 50BP. The IFN gamma standard curve was prepared as described in Section 10. Raw data values are shown in the table. Background-subtracted data values (mean +/- SD) are graphed.

16. Calibration

This immunoassay is calibrated against a highly purified human IFN gamma. The NIBSC/WHO unclassified purified human IFN gamma preparation 87/586 was evaluated in this kit.

The dose response curve of the unclassified standard IFN gamma parallels the SimpleStep standard curve. To convert sample values obtained with the SimpleStep Human IFN gamma kit to approximate NIBSC 87/586 units, use the equation below.

NIBSC (87/586) approximate value (IU/mL) = 0.01113 x SimpleStep Human IFN gamma value (pg/mL).

17. Typical Sample Values

SENSITIVITY –
The MDD was determined by calculating the mean of zero standard replicates and adding 2 standard deviations then extrapolating the corresponding concentration.

Sample Diluent Buffer	n=	Minimal Detectable Dose
Sample Diluent NS	16	2.0 pg/mL
Sample Diluent 50BP	15	4.0 pg/mL

RECOVERY –

Three concentrations of IFN gamma recombinant protein were spiked in duplicate to the indicated biological matrix to evaluate signal recovery in the working range of the assay.

Sample Type	Average % Recovery	Range (%)
Neat Serum	128	122 – 133
Neat Plasma – Citrate	121	120 – 121
Neat Plasma – EDTA	115	109 – 120
50% Saliva	90	87 – 92
12% PHA-M Stimulated PBMC Cell Culture Supernatant	100	98 – 101
50% Cell Culture Media*	94	91 – 97

*Media is RPMI 1640 containing 10% fetal calf serum.

Linearity of Dilution

Linearity of dilution is determined based on interpolated values from the standard curve. Linearity of dilution defines a sample concentration interval in which interpolated target concentrations are directly proportional to sample dilution.

Recombinant IFN gamma was spiked into serum, plasma, and saliva samples and diluted in a 2-fold dilution series. Serum and plasma samples are diluted in Sample Diluent 50BP. Saliva samples are diluted in Sample Diluent NS.

Native IFN gamma was measured in PHA-M (46hr) treated PBMC cell supernatant (SN) samples in a 2-fold dilution series. Sample dilutions are made in Sample Diluent NS.

Dilution Factor	Interpolated value	100% Human Serum	100% Human Plasma (Citrate)	100% Human Plasma (EDTA)	50% Human Saliva	50% PHA-M PBMC SN
Undiluted	pg/mL	1149	1021	1070	399.0	537.3
	% Expected value	100	100	100	100	100
2	pg/mL	512.4	495.8	508.4	210.8	261.6
	% Expected value	89	97	95	106	97
4	pg/mL	264.7	255.4	255.6	117.9	137.2
	% Expected value	92	100	96	118	102
8	pg/mL	127.8	125.1	125.9	58.75	71.49
	% Expected value	89	98	94	118	106
16	pg/mL	64.71	61.02	64.34	28.63	35.93
	% Expected value	90	96	96	115	107

Neat pooled serum, neat pooled plasma (EDTA, Citrate), and 50% pooled saliva samples from healthy donors was measured in duplicate. All values were below the detectable range of the assay.

PRECISION –

Mean coefficient of variations of interpolated values of IFN gamma from three concentrations of PHA-M treated PBMC cell culture supernatant within the working range of the assay.

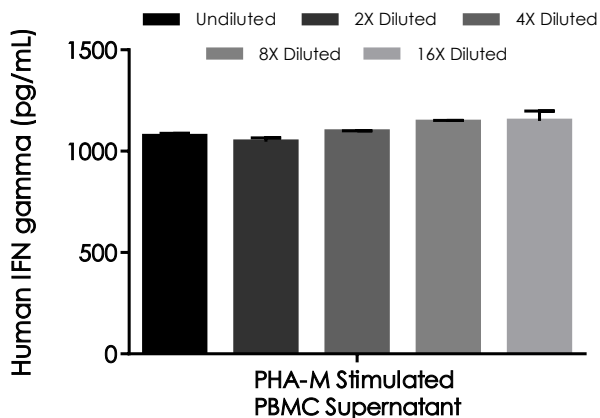|	Intra-Assay	Inter-Assay
n =	5	3
CV (%)	2.6	5.3

Figure 3. Interpolated concentrations of native IFN gamma in human PHA-M stimulated PBMC cell culture supernatant samples. The concentrations of IFN gamma were measured in duplicates, interpolated from the IFN gamma standard curves and corrected for sample dilution. Undiluted samples are as follows: PBMC supernatant 50%. The interpolated dilution factor corrected values are plotted (mean +/- SD, n=2). The mean IFN gamma concentration was determined to be 1102 pg/mL in neat PHA-M stimulated PBMC cell culture supernatant.
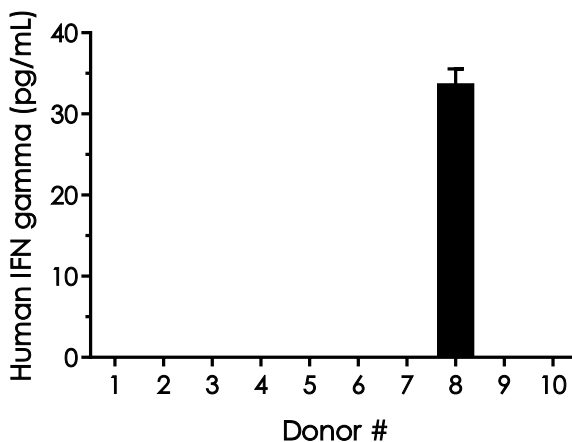
Figure 4. Serum from ten individual healthy human female donors was measured in duplicate. Interpolated dilution factor corrected values are plotted (mean +/- SD, n=2). The mean IFN gamma concentration was determined to be 33.5 pg/mL with a range of non-detectable – 33.5 pg/mL.
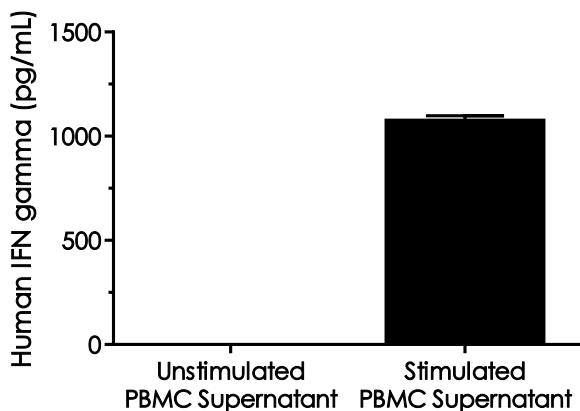
Figure 5. Comparison of Interferon gamma in unstimulated and PHA-M stimulated PBMC cell culture supernatants. Human PBMCs were cultured for 46 hours in the presence or absence of 1.5% PHA-M. The concentrations of Interferon gamma were measured in three different dilutions of the supernatant samples in duplicates and interpolated from the Interferon gamma standard curve. The interpolated values are plotted (mean +/- SD, n=3). The mean Interferon gamma concentration was determined to be 1,073 pg/mL in PHA-M stimulated PBMC cell culture supernatant, and undetectable in unstimulated PBMC cell culture supernatant and RPMI1640 media containing 10% FBS (not shown).

18. Assay Specificity

This kit recognizes both native and recombinant human IFN gamma protein in serum, plasma (citrate and EDTA), saliva, and cell culture supernatant samples only.

This kit is not compatible with plasma (heparin) samples.

Urine, milk, cell and tissue extract samples have not been tested with this kit.

19. Species Reactivity

This kit recognizes human IFN gamma protein.

Other species reactivity was determined by measuring IFN gamma recombinant protein of various species within the working range of the standard curve. This kit is reactive with Rhesus macaque IFN gamma recombinant protein. No Rhesus macaque biological samples have been tested.

This kit does not react with the following species:
– Mouse
– Rat

Please contact our Technical Support team for more information.

20. Troubleshooting

Problem	Reason	Solution
Poor standard curve	Inaccurate Pipetting	Check pipettes
	Improper standard dilution	Prior to opening, briefly spin the stock standard tube and dissolve the powder thoroughly by gentle mixing
Low Signal	Incubation times too brief	Ensure sufficient incubation times; increase to 2 or 3 hour standard/sample incubation
	Inadequate reagent volumes or improper dilution	Check pipettes and ensure correct preparation
	Incubation times with TMB too brief	Ensure sufficient incubation time until blue color develops prior addition of Stop solution
Large CV	Plate is insufficiently washed	Review manual for proper wash technique. If using a plate washer, check all ports for obstructions.
	Contaminated wash buffer	Prepare fresh wash buffer
Low sensitivity	Improper storage of the ELISA kit	Store your reconstituted standards at -80°C, all other assay components 4°C. Keep TMB Development Solution protected from light.
Precipitate in Diluent	Precipitation and/or coagulation of components within the Diluent.	Precipitate can be removed by gently warming the Diluent to 37°C.

Technical Support

For all technical or commercial enquiries please go to:

www.abcam.com/contactus
www.abcam.cn/contactus (China)
www.abcam.co.jp/contactus (Japan)