Human IFN gamma High Sensitivity ELISA Kit

Enzyme-linked Immunosorbent Assay for quantitative detection of human IFNγ

Catalog Number BMS228HS

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WARNING! Read the Safety Data Sheets (SDSs) and follow the handling instructions. Wear appropriate protective eyewear, clothing, and gloves. Safety Data Sheets (SDSs) are available from **thermofisher.com/support**.

Product description

The Human IFN gamma High Sensitivity ELISA Kit is an enzyme-linked immunosorbent assay for the quantitative detection of human IFNγ.

Summary

IFNγ, also called Type II interferon, is a homodimeric glycoprotein containing approximately 21 to 24 kD subunits. The human IFNγ gene, situated on chromosome 12, contains three introns; the four exons code for a polypeptide of 166 amino acids, 20 of which constitute the signal peptide.

In contrast to IFNα and IFNβ synthesis, which can occur in any cell, production of IFNγ is a function of T cells and NK cells. All IFNγ inducers activate T cells either in a polyclonal (mitogens or antibodies) or in a clonally restricted, antigen-specific, manner.

IFNγ is produced during infection by T cells of the cytotoxic/suppressor phenotype (CD8) and by a subtype of helper T cells, the Th1 cells. Th1 cells secrete IL-2, IL-3, TNFβ and IFNγ, whereas Th2 cells mainly produce IL-3, IL-4, IL-5, and IL-10, but little or no IFNγ. IFNγ preferentially inhibits the proliferation of Th2 but not Th1 cells, indicating that the presence of IFNγ during an immune response will result in the preferential proliferation of Th1 cells.

Type II IFN or IFNγ is a lymphokine that displays no molecular homology with type I IFN, but shares some important biologic activities. Specifically, IFNγ induces an anti-viral state and is anti-proliferative. In addition, IFNγ has several properties related to immunoregulation.

1. IFNγ is a potent activator of mononuclear phagocytes, e.g. IFNγ stimulates the expression of Mac-1, augments endocytosis and phagocytosis by monocytes, and activates macrophages to kill tumor cells by releasing reactive oxygen intermediates and TNFγ.
2. IFNγ induces or augments the expression of MHC antigens on macrophages, T and B cells and some tumor cell lines.
3. On T and B cells IFNγ promotes differentiation. It enhances proliferation of activated B cells and can act synergistically with IL-2 to increase immunoglobulin light-chain synthesis. IFNγ is one of the natural B-cell differentiation factors.
4. Finally, IFNγ activates neutrophils, NK cells and vascular endothelial cells.

The role of IFNγ as a disease marker has been demonstrated for a number of different pathological situations.

For literature update refer to our website.

Principles of the test

An anti-human IFNγ coating antibody is adsorbed onto microwells.

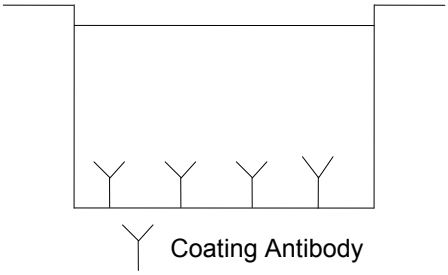

Fig. 1 Coated microwell

Human IFNγ present in the sample or standard binds to antibodies adsorbed to the microwells. A biotin-conjugated anti-human IFNγ antibody is added and binds to human IFNγ captured by the first antibody.

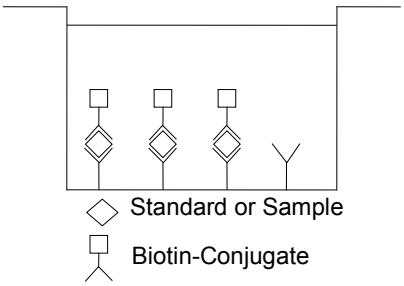

Fig. 2 First incubation

Following incubation unbound biotin-conjugated anti-human IFNγ antibody is removed during a wash step. Streptavidin-HRP is added and binds to the biotin-conjugated anti-human IFNγ antibody.

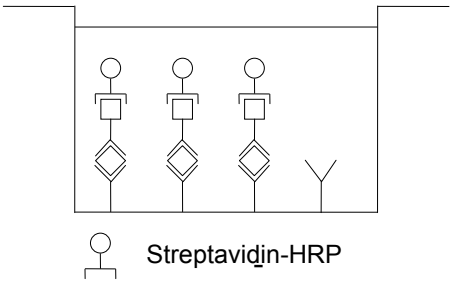

Fig. 3 Second incubation

Following incubation unbound Streptavidin-HRP is removed during a wash step, and amplification reagent I (Biotinyl-Tyramide) is added to the wells.

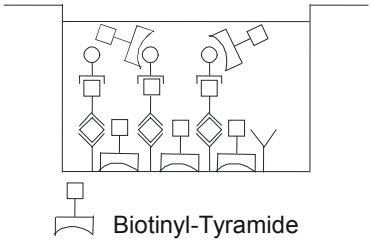

Fig. 4 Third incubation

Following incubation unbound amplification reagent I is removed during a wash step and amplification reagent II (Streptavidin-HRP) is added.

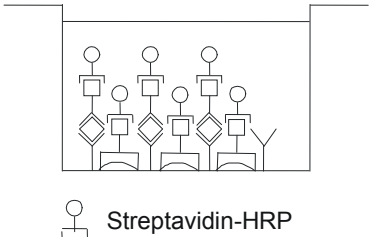

Fig. 5 Fourth incubation

Following incubation unbound amplification reagent II is removed during a wash step and substrate solution reactive with HRP is added.

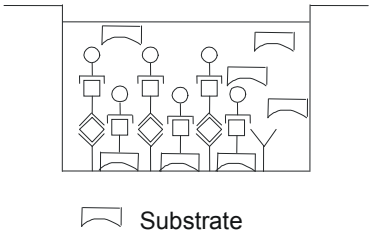

Fig. 6 Fifth incubation

A colored product is formed in proportion to the amount of human IFNγ present in the sample or standard. The reaction is terminated by addition of acid and absorbance is measured at 450 nm. A standard curve is prepared from 7 human IFNγ standard dilutions and human IFNγ sample concentration determined.

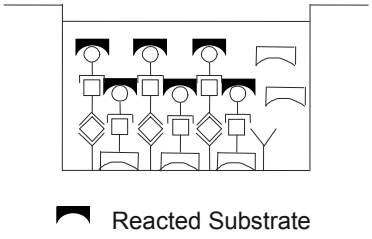

Fig. 7 Stop reaction

Principle of amplification reaction

The amplification reaction is based upon PerkinElmer Life Sciences TSA™ technology.

Amplification reagent I contains biotinyl-tyramide. HRP converts multiple biotinyl-tyramide molecules into highly reactive derivates (free radicals). These free radicals bind covalently to any protein in the well.

Thus, the amount of reacted biotinyl-tyramide is proportional to the amount of HRP in the well.

Following incubation unbound biotinyl-tyramide is removed during a wash step. Amplification reagent II contains Streptavidin-HRP, which binds to the biotin sites created during the biotinyl-tyramide reaction, thus multiplying the HRP molecules available at the surface for the substrate reaction.

Reagents provided

1 aluminum pouch with a Microwell Plate (12 strips of 8 wells each) coated with monoclonal antibody to human IFNγ

1 vial (100 µL) Biotin-Conjugate anti-human IFNγ monoclonal antibody

1 vial (150 µL) Streptavidin-HRP

2 vials human IFNγ Standard lyophilized, 200 ng/mL upon reconstitution

1 vial (12 mL) Sample Diluent

Note: In some, very rare cases, an insoluble precipitate of stabilizing protein has been seen in the Sample Diluent vial. This precipitate does not interfere in any way with the performance of the test and can thus be ignored.

1 vial (5 mL) Assay Buffer Concentrate 20x (PBS with 1% Tween 20 and 10% BSA)

1 vial (7 mL) Amplification Diluent Concentrate (2x)

1 vial (75 µL) Amplification Reagent I

Note: reagent contains ethyl alcohol

2 vials (15 µL) Amplification Reagent II

2 bottles (50 mL) Wash Buffer Concentrate 20x (PBS with 1% Tween 20)

1 vial (15 mL) Substrate Solution

1 vial (15 mL) Stop Solution (1M Phosphoric acid)

8 Adhesive Films

Storage instructions – ELISA kit

Store kit reagents between 2° and 8°C. Immediately after use remaining reagents should be returned to cold storage (2° to 8°C). Expiry of the kit and reagents is stated on labels.

Expiry of the kit components can only be guaranteed if the components are stored properly, and if, in case of repeated use of one component, this reagent is not contaminated by the first handling.

Sample collection and storage instructions

Cell culture supernatant, serum, and plasma (EDTA, citrate and heparin) were tested with this assay. Other biological samples might be suitable for use in the assay. Remove serum or plasma from the clot or cells as soon as possible after clotting and separation. Samples containing a visible precipitate must be clarified prior to use in the assay. Do not use grossly hemolyzed or lipemic samples.

Samples should be aliquoted and must be stored frozen at –20°C to avoid loss of bioactive human IFNγ. If samples are to be run within 24 hours, they may be stored at 2–8°C (refer to "Sample stability" on page 7). Avoid repeated freeze-thaw cycles. Prior to assay, the frozen sample should be brought to room temperature slowly and mixed gently.

Materials required but not provided

- 5 mL and 10 mL graduated pipettes
- 5 µL to 1000 µL adjustable single channel micropipettes with disposable tips
- 50 µL to 300 µL adjustable multichannel micropipette with disposable tips
- Multichannel micropipette reservoir
- Beakers, flasks, cylinders necessary for preparation of reagents
- Device for delivery of wash solution (multichannel wash bottle or automatic wash system)
- Microplate shaker
- Microwell strip reader capable of reading at 450 nm (620 nm as optional reference wave length)
- Glass-distilled or deionized water
- Statistical calculator with program to perform regression analysis

Precautions for use

- All chemicals should be considered as potentially hazardous. We therefore recommend that this product is handled only by those persons who have been trained in laboratory techniques and that it is used in accordance with the principles of good laboratory practice. Wear suitable protective clothing such as laboratory overalls, safety glasses, and gloves. Care should be taken to avoid contact with skin or eyes. In the case of contact with skin or eyes wash immediately with water. See material safety data sheet(s) and/or safety statement(s) for specific advice.
- Reagents are intended for research use only and are not for use in diagnostic or therapeutic procedures.
- Do not mix or substitute reagents with those from other lots or other sources.
- Do not use kit reagents beyond expiration date on label.
- Do not expose kit reagents to strong light during storage or incubation.
- Do not pipet by mouth.
- Do not eat or smoke in areas where kit reagents or samples are handled.
- Avoid contact of skin or mucous membranes with kit reagents or samples.
- Rubber or disposable latex gloves should be worn while handling kit reagents or samples.
- Avoid contact of substrate solution with oxidizing agents and metal.
- Avoid splashing or generation of aerosols.
- To avoid microbial contamination or cross-contamination of reagents or samples that may invalidate the test, use disposable pipette tips and/or pipettes.
- Use clean, dedicated reagent trays for dispensing the conjugate and substrate reagent.
- Exposure to acid inactivates the conjugate.
- Glass-distilled water or deionized water must be used for reagent preparation.
- Substrate solution must be at room temperature prior to use.
- Decontaminate and dispose samples and all potentially contaminated materials as if they could contain infectious agents. The preferred method of decontamination is autoclaving for a minimum of 1 hour at 121.5°C.
- Liquid wastes not containing acid and neutralized waste may be mixed with sodium hypochlorite in volumes such that the final mixture contains 1.0% sodium hypochlorite. Allow 30 minutes for effective decontamination. Liquid waste containing acid must be neutralized prior to the addition of sodium hypochlorite.

Preparation of reagents

1. Buffer concentrates should be brought to room temperature and should be diluted before starting the test procedure.
2. If crystals have formed in buffer concentrates, warm them gently until crystals have completely dissolved.

Wash buffer (1x)

1. If crystals have formed in the Wash Buffer Concentrate (20x), warm it gently until they have completely dissolved.
2. Pour entire contents (50 mL) of the Wash Buffer Concentrate (20x) into a clean 1000 mL graduated cylinder. Bring to final volume of 1000 mL with glass-distilled or deionized water. Mix gently to avoid foaming.
3. Transfer to a clean wash bottle and store at 2° to 25°C. Please note that Wash Buffer is stable for 30 days.
4. Wash Buffer (1x) may also be prepared as needed according to the following table:

Number of Strips	Wash Buffer Concentrate (mL)	Distilled Water (mL)
1 - 6	25	475
1 - 12	50	950

Assay buffer (1x)

1. Pour the entire contents (5 mL) of the Assay Buffer Concentrate (20x) into a clean 100 mL graduated cylinder. Bring to final volume of 100 mL with distilled water. Mix gently to avoid foaming.
2. Store at 2° to 8°C. Please note that the Assay Buffer (1x) is stable for 30 days.
3. Assay Buffer (1x) may also be prepared as needed according to the following table:

Number of Strips	Assay Buffer Concentrate (mL)	Distilled Water (mL)
1 - 6	2.5	47.5
1 - 12	5.0	95.0

Biotin-Conjugate

Note: The Biotin-Conjugate should be used within 30 minutes after dilution.

Make a 1:100 dilution of the concentrated Biotin-Conjugate solution with Assay Buffer (1x) in a clean plastic tube as needed according to the following table:

Number of Strips	Biotin-Conjugate (mL)	Assay Buffer (1x) (mL)
1 - 6	0.03	2.97
1 - 12	0.06	5.94

Streptavidin-HRP

Note: The Streptavidin-HRP should be used within 30 minutes after dilution.

Make a 1:400 dilution of the concentrated Streptavidin-HRP solution with Assay Buffer (1x) in a clean plastic tube as needed according to the following table:

Number of Strips	Streptavidin-HRP (mL)	Assay Buffer (1x) (mL)
1 - 6	0.015	5.985
1 - 12	0.03	11.97

Human IFNγ standard

1. Reconstitute human IFNγ standard by addition of distilled water. Reconstitution volume is stated on the label of the standard vial. Swirl or mix gently to insure complete and homogeneous solubilization (concentration of reconstituted standard = 200 ng/mL).
2. Allow the standard to reconstitute for 10-30 minutes. Mix well prior to making dilutions.
3. The standard has to be used immediately after reconstitution and cannot be stored.
4. The concentrated human IFNγ standard must be diluted 1:10.000 with Assay Buffer (1x) just prior to use in a clean plastic test tube according to the following dilution scheme:
   - **Dilution I:** 10 µL concentrated human IFNγ standard + 990 µL Assay Buffer (1x).
   - **Dilution II:** 10 µL of dilution I + 990 µL Assay Buffer (1x).

   Shake gently to mix (concentration of standard = 20 pg/mL).
5. Standard dilutions can be prepared directly on the microwell plate (see "Test protocol" on page 4) or alternatively in tubes (see "External standard dilution" on page 3).

External standard dilution

1. Label 7 tubes, one for each standard point: S1, S2, S3, S4, S5, S6, S7.
2. Prepare 1:2 serial dilutions for the standard curve as follows:
3. Pipette 225 µL of Sample Diluent into each tube.
4. Pipette 225 µL of diluted standard (concentration of standard = 20 pg/mL) into the first tube, labeled S1, and mix (concentration of standard 1 = 10 pg/mL).

5. Pipette 225 µL of this dilution into the second tube, labeled S2, and mix thoroughly before the next transfer.
6. Repeat serial dilutions 5 more times thus creating the points of the standard curve (see Figure 8).

   Sample Diluent serves as blank.

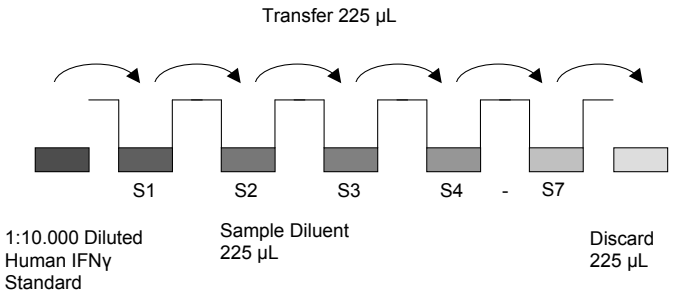

Fig. 8 Dilute standards - tubes

Amplification diluent (1x)

Preparation of Amplification Diluent (1x) has to be done immediately prior to use. Make a 1:2 dilution of the concentrated Amplification Diluent (2x) as needed according to the following table:

Number of Strips	Amplification Diluent (2x) (mL)	Distilled Water (mL)
1 - 6	3	3
1 - 12	6	6

Amplification solution I

1. Prepare Amplification Solution I immediately prior to application on the plate.
2. Dilute Amplification Reagent I in Amplification Diluent (1x) as indicated in the Certificate of Analysis.
3. Discard immediately any prediluted Amplification Solution I after usage.

Amplification solution II

1. Prepare Amplification Solution II immediately prior to application on the plate.
2. Centrifuge vial for a few seconds in a microcentrifuge before opening to collect liquid trapped in the lid.
3. Dilute Amplification Reagent II in Assay Buffer (1x) as as indicated in the Certificate of Analysis.
4. Discard immediately any prediluted Amplification Solution II after usage.

Test protocol

IMPORTANT!

- Because this ELISA is a high sensitivity system, it is extremely important to stick exactly to the manual (washing procedure, chronology of and preparation of solutions, incubation time) to obtain optimal test performance.
- Amplification Solutions must be prepared immediately prior to application on the plate! It is extremely important to wash the wells properly to obtain a good test performance.
- Shaking is absolutely necessary for an optimal test performance. Protect microwell plate from light during incubation steps

1. Determine the number of microwell strips required to test the desired number of samples plus appropriate number of wells needed for running blanks and standards. Each sample, standard, blank and optional control sample should be assayed in duplicate. Remove extra microwell strips from holder and store in foil bag with the desiccant provided at 2°-8°C sealed tightly.
2. Wash the microwell strips twice with exactly 400 µL Wash Buffer per well with thorough aspiration of microwell contents between washes. Allow the Wash Buffer to sit in the wells for about 10 – 15 seconds before aspiration. Soaking is highly recommended between the washes to obtain a good test performance! Take care not to scratch the surface of the microwells.

   After the last wash step, empty wells and tap microwell strips on absorbent pad or paper towel to remove excess Wash Buffer. Use the microwell strips immediately after washing. Do not allow wells to dry.
3. Standard dilution on the microwell plate (Alternatively the standard dilution can be prepared in tubes - see "External standard dilution" on page 3.):

   Add 100 µL of Sample Diluent in duplicate to all standard wells. Pipette 100 µL of prepared standard (see "Human IFNγ standard" on page 3, concentration = 20 pg/mL) in duplicate into well A1 and A2 (see Table 1). Mix the contents of wells A1 and A2 by repeated aspiration and ejection (concentration of standard 1, S1 = 10 pg/mL), and transfer 100 µL to wells B1 and B2, respectively (see Figure 9). Take care not to scratch the inner surface of the microwells. Continue this procedure 5 times, creating two rows of human IFNγ standard dilutions ranging from 10.00 to 0.16 pg/mL. Discard 100 µL of the contents from the last microwells (G1, G2) used.

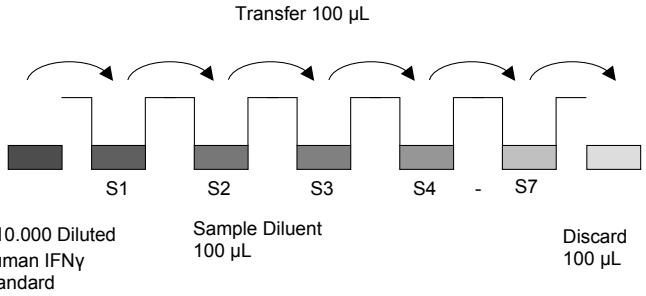

Fig. 9 Dilute standards - microwell plate

Table 1 Example of the arrangement of blanks, standards and samples in the microwell strips.

	1	2	3	4
A	Standard 1 10.00 pg/mL	Standard 1 10.00 pg/mL	Sample 1	Sample 1
B	Standard 2 5.00 pg/mL	Standard 2 5.00 pg/mL	Sample 2	Sample 2
C	Standard 3 2.50 pg/mL	Standard 3 2.50 pg/mL	Sample 3	Sample 3
D	Standard 4 1.25 pg/mL	Standard 4 1.25 pg/mL	Sample 4	Sample 4
E	Standard 5 0.63 pg/mL	Standard 5 0.63 pg/mL	Sample 5	Sample 5
F	Standard 6 0.31 pg/mL	Standard 6 0.31 pg/mL	Sample 6	Sample 6
G	Standard 7 0.16 pg/mL	Standard 7 0.16 pg/mL	Sample 7	Sample 7
H	Blank	Blank	Sample 8	Sample 8

   In case of an external standard dilution (see "External standard dilution" on page 3.), pipette 100 µL of these standard dilutions (S1 - S7) in the standard wells according to Table 1.
4. Add 100 µL of Sample Diluent in duplicate to the blank wells.
5. Add 50 µL of Sample Diluent to the sample wells.
6. Add 50 µL of each sample in duplicate to the sample wells.
7. Prepare Biotin-Conjugate (see "Biotin-Conjugate" on page 3).
8. Add 50 µL of Biotin-Conjugate to all wells.

9. Cover with an adhesive film and incubate at room temperature (18° to 25°C) for 2 hours on a microplate shaker. (Shaking is absolutely necessary for an optimal test performance.)
10. Prepare Streptavidin-HRP (refer to "Streptavidin-HRP" on page 3).
11. Remove adhesive film and empty wells. Wash microwell strips 6 times according to point 2. of the test protocol. Proceed immediately to the next step.
12. Add 100 µL of diluted Streptavidin-HRP to all wells, including the blank wells.
13. Cover with an adhesive film and incubate at room temperature (18° to 25°C) for 1 hour on a microplate shaker. (Shaking is absolutely necessary for an optimal test performance.)
14. Prepare Amplification Solution I diluted in Amplification Diluent (see "Amplification solution I" on page 4) immediately prior to use.
15. Remove adhesive film and empty wells. Wash microwell strips 6 times according to point 2. of the test protocol. Proceed immediately to the next step.
16. Add 100 µL of Amplification Solution I to all wells, including the blank wells.
17. Cover with an adhesive film and incubate at room temperature (18° to 25°C) for exactly 15 minutes on a microplate shaker. (Shaking is absolutely necessary for an optimal test performance.)
18. Prepare Amplification Solution II diluted in Assaybuffer (see "Amplification solution II" on page 4) immediately prior to use.
19. Remove adhesive film and empty wells. Wash microwell strips 6 times according to point 2. of the test protocol. Proceed immediately to the next step.
20. Add 100 µL of Amplification Solution II to all wells, including the blank wells.
21. Cover with an adhesive film and incubate at room temperature (18° to 25°C) for exactly 30 minutes on a microplate shaker. (Shaking is absolutely necessary for an optimal test performance.)
22. Remove adhesive film and empty wells. Wash microwell strips 6 times according to point 2. of the test protocol. Proceed immediately to the next step.
23. Pipette 100 µL of TMB Substrate Solution to all wells.
24. Incubate the microwell strips at room temperature (18° to 25°C) for about 10-20 minutes. Avoid direct exposure to intense light.

    The color development on the plate should be monitored and the substrate reaction stopped (see next point of this protocol) before positive wells are no longer properly recordable. Determination of the ideal time period for color development has to be done individually for each assay.

    It is recommended to add the stop solution when the highest standard has developed a dark blue color. Alternatively the color development can be monitored by the ELISA reader at 620 nm. The substrate reaction should be stopped as soon as Standard 1 has reached an OD of 0.9 – 0.95.
25. Stop the enzyme reaction by quickly pipetting 100 µL of Stop Solution into each well. It is important that the Stop Solution is spread quickly and uniformly throughout the microwells to completely inactivate the enzyme. Results must be read immediately after the Stop Solution is added or within one hour if the microwell strips are stored at 2 - 8°C in the dark.
26. Read absorbance of each microwell on a spectro-photometer using 450 nm as the primary wave length (optionally 620 nm as the reference wave length; 610 nm to 650 nm is acceptable). Blank the plate reader according to the manufacturer's instructions by using the blank wells. Determine the absorbance of both the samples and the standards.

Note: In case of incubation without shaking the obtained O.D. values may be lower than indicated below. Nevertheless the results are still valid.

Calculation of results

- Calculate the average absorbance values for each set of duplicate standards and samples. Duplicates should be within 20 percent of the mean value.
- Create a standard curve by plotting the mean absorbance for each standard concentration on the ordinate against the human IFNγ concentration on the abscissa. Draw a best fit curve through the points of the graph (a 5-parameter curve fit is recommended).
- To determine the concentration of circulating human IFNγ for each sample, first find the mean absorbance value on the ordinate and extend a horizontal line to the standard curve. At the point of intersection, extend a vertical line to the abscissa and read the corresponding human IFNγ concentration.
- If instructions in this protocol have been followed samples have been diluted 1:2 (50 µL sample + 50 µL Sample Diluent). Thus concentrations read from the standard curve must be multiplied by the dilution factor (x 2).
- Calculation of samples with a concentration exceeding standard 1 may result in incorrect, low human IFNγ levels. Such samples require further external predilution according to expected human IFNγ values with Sample Diluent in order to precisely quantitate the actual human IFNγ level.
- It is suggested that each testing facility establishes a control sample of known human IFNγ concentration and runs this additional control with each assay. If the values obtained are not within the expected range of the control, the assay results may be invalid.
- A representative standard curve is shown in Figure 10. This curve cannot be used to derive test results. Each laboratory must prepare a standard curve for each group of microwell strips assayed.

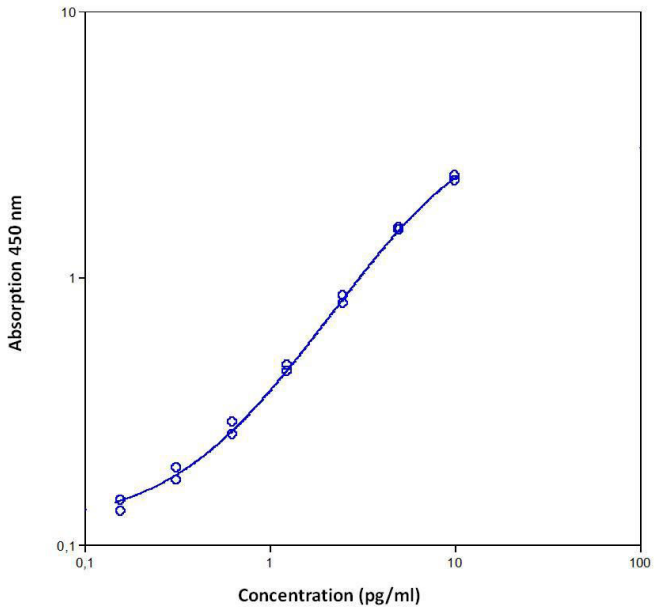

Fig. 10 Representative standard curve for human IFNγ ELISA. Human IFNγ was diluted in serial 2-fold steps in Sample Diluent. Do not use this standard curve to derive test results. A standard curve must be run for each group of microwell strips assayed.

Table 2 Typical data using the human IFNγ ELISA

Measuring wavelength: 450 nm

Reference wavelength: 620 nm

Standard	Human IFNγ Concentration (pg/mL)	O.D. at 450 nm	Mean O.D. at 450 nm	C.V. (%)
1	10.00	2.411 2.320	2.366	1.9
2	5.00	1.540 1.503	1.522	1.2
3	2.50	0.804 0.861	0.832	3.4
4	1.25	0.444 0.471	0.457	2.9
5	0.63	0.258 0.288	0.273	5.6
6	0.31	0.174 0.194	0.184	5.6
7	0.16	0.134 0.147	0.141	4.8
Blank	0.00	0.092 0.089	0.091	1.4

The OD values of the standard curve may vary according to the conditions of assay performance (e.g. operator, pipetting technique, washing technique or temperature effects). Furthermore shelf life of the kit may affect enzymatic activity and thus color intensity. Values measured are still valid.

Limitations

- Since exact conditions may vary from assay to assay, a standard curve must be established for every run.
- Bacterial or fungal contamination of either screen samples or reagents or cross-contamination between reagents may cause erroneous results.
- Disposable pipette tips, flasks or glassware are preferred, reusable glassware must be washed and thoroughly rinsed of all detergents before use.
- Improper or insufficient washing at any stage of the procedure will result in either false positive or false negative results. Empty wells completely before dispensing fresh wash solution, fill with Wash Buffer as indicated for each wash cycle and do not allow wells to sit uncovered or dry for extended periods.
- The use of radioimmunotherapy has significantly increased the number of subjects with human anti-mouse IgG antibodies (HAMA). HAMA may interfere with assays utilizing murine monoclonal antibodies leading to both false positive and false negative results. Serum samples containing antibodies to murine immunoglobulins can still be analyzed in such assays when murine immunoglobulins (serum, ascitic fluid, or monoclonal antibodies of irrelevant specificity) are added to the sample.

Performance characteristics

Sensitivity

The limit of detection of human IFNγ defined as the analyte concentration resulting in an absorbance significantly higher than that of the dilution medium (mean plus 2 standard deviations) was determined to be 0.06 pg/mL (mean of 6 independent assays).

Reproducibility

Intra-assay

Reproducibility within the assay was evaluated in 3 independent experiments. Each assay was carried out with 6 replicates of 7 serum samples containing different concentrations of human IFNγ. 2 standard curves were run on each plate. Data below show the mean human IFNγ concentration and the coefficient of variation for each sample (see Table 3). The calculated overall intra-assay coefficient of variation was 6.8%.

Table 3 The mean human IFNγ concentration and the coefficient of variation for each sample

Sample	Experiment	Mean Human IFNγ Concentration (pg/mL)	Coefficient of Variation (%)
1	1 2 3	36.20 37.90 34.93	8.1 7.7 6.9
2	1 2 3	28.73 25.88 26.24	5.6 6.0 4.1
3	1 2 3	15.61 14.53 14.49	9.0 7.0 3.9
4	1 2 3	11.42 12.54 13.33	7.6 5.8 9.2
5	1 2 3	5.63 4.21 5.58	10.5 11.0 6.6
6	1 2 3	3.13 3.02 2.81	3.9 8.0 1.2
7	1 2 3	2.29 1.74 2.50	4.8 7.0 8.4

Inter-assay

Assay to assay reproducibility within one laboratory was evaluated in 2 independent experiments. Each assay was carried out with 6 replicates of 7 serum samples containing different concentrations of human IFNγ. 2 standard curves were run on each plate. Data below show the mean human IFNγ concentration and the coefficient of variation calculated on 18 determinations of each sample (see Table 4). The calculated overall inter-assay coefficient of variation was 7.1%.

Table 4 The mean human IFNγ concentration and the coefficient of variation of each sample

Sample	Mean Human IFNγ Concentration (pg/mL)	Coefficient of Variation (%)
1	36.34	3.3
2	26.95	4.7
3	14.88	3.5
4	12.43	6.3
5	5.14	12.8
6	2.99	4.4
7	2.18	14.6

Spike recovery

The spike recovery was evaluated by spiking four levels of human IFNγ into pooled normal human serum samples. Recoveries were determined in 2 independent experiments with 8 replicates each. The amount of endogenous human IFNγ in unspiked serum was subtracted from the two spike values. The recovery ranged from 75–115% with an overall mean recovery of 89%.

Dilution parallelism

3 serum samples with different levels of human IFNγ were analyzed at serial 2-fold dilutions with 4 replicates each. The recovery ranged from 80–124% with an overall recovery of 97%.

Sample	Dilution	Human IFNγ (pg/mL)		Recovery of expected concentration (%)
		Expected concentration	Observed concentration	
1	1:2	–	19.6	–
	1:4	9.8	10.3	105
	1:8	4.9	5.0	102
	1:16	2.5	2.4	97
2	1:2	–	22.1	–
	1:4	11.0	10.4	94
	1:8	5.5	6.8	124
	1:16	2.8	2.5	90
3	1:2	–	41.1	–
	1:4	20.6	16.5	80
	1:8	8.3	7.7	93
	1:16	3.8	3.5	91

Sample stability

Freeze-Thaw stability

Aliquots of serum and cell culture supernatant samples (spiked or unspiked) were stored at -20°C and thawed 5 times, and the human IFNγ levels determined. There was no significant loss of human IFNγ immuno-reactivity detected by 1 cycle of freezing and thawing.

Further freeze-thaw cycles gave rise to about 30 % loss of IFNγ immunoreactivity.

Storage stability

Aliquots of serum and cell culture supernatant samples (spiked or unspiked) were stored at –20°C, 2–8°C, room temperature, and at 37°C, and the human IFNγ level determined after 24 hours. There was no significant loss of human IFNγ immunoreactivity detected during storage at –20°C, 2–8°C., and room temperature.

A significant loss of human IFNγ immunoreactivty (50%) was detected during storage at 37°C after 24 hours.

Comparison of serum and plasma

From 2 individuals, serum as well as EDTA, citrate and heparin plasma obtained at the same time point, were evaluated. Human IFNγ concentrations were not significantly different and therefore all these body fluids are suitable for the assay. It is nevertheless highly recommended to assure the uniformity of blood preparations.

Specificity

The interference of circulating factors of the immune system was evaluated by spiking these proteins at physiologically relevant concentrations into a human IFNγ positive serum. No cross-reactivity was detected.

Expected values

A panel of 22 serum samples from randomly selected apparently healthy donors (males and females) was tested for human IFNγ. The detected human IFNγ sera levels ranged between 0.15 and 168.00 pg/mL with a mean level of 10.40 pg/mL and a standard deviation of 40.00 pg/mL.

Calibration

This immunoassay is calibrated with highly purified recombinant human IFNγ, which has been evaluated against the International Reference Standard NIBSC 82/587 and has been shown to be equivalent.

NIBSC 82/587 is quantitated in International Units (IU), 1IU corresponding to 50 pg human IFNγ.

Reagent preparation summary

Wash buffer (1x)

Add Wash Buffer Concentrate 20x (50 mL) to 950 mL distilled water.

Number of Strips	Wash Buffer Concentrate (mL)	Distilled Water (mL)
1 - 6	25	475
1 - 12	50	950

Assay buffer (1x)

Add Assay Buffer Concentrate 20x (5 mL) to 95 mL distilled water.

Number of Strips	Assay Buffer Concentrate (mL)	Distilled Water (mL)
1 - 6	2.5	47.5
1 - 12	5.0	95.0

Biotin-Conjugate

Make a 1:100 dilution of Biotin-Conjugate in Assay Buffer (1x):

Number of Strips	Biotin-Conjugate (mL)	Assay Buffer (1x) (mL)
1 - 6	0.03	2.97
1 - 12	0.06	5.94

Streptavidin-HRP

Make a 1:400 dilution of Streptavidin-HRP in Assay Buffer (1x):

Number of Strips	Streptavidin-HRP (mL)	Assay Buffer (1x) (mL)
1 - 6	0.015	5.985
1 - 12	0.03	11.97

Human IFNγ standard

1. Reconstitute lyophilized human IFNγ standard with distilled water. (Reconstitution volume is stated on the label of the standard vial.)
2. The concentrated human IFNγ standard must be diluted 1:10.000 with Assay Buffer (1x).

Amplification diluent (1x)

Preparation of Amplification Diluent (1x) has to be done immediately prior to use.

Number of Strips	Amplification Diluent (2x) (mL)	Distilled Water (mL)
1 - 6	3	3
1 - 12	6	6

Amplification solution I

Dilute Amplification Reagent I in Amplification Diluent (1x) immediately prior to application on the plate as indicated in the Certificate of Analysis.

Amplification solution II

Centrifuge vial for a few seconds in a micro-centrifuge before opening to collect liquid trapped in the lid. Dilute Amplification Solution II in Assay Buffer (1x) immediately prior to application on the plate as indicated in the Certificate of Analysis.

Test protocol summary

Note: Prepare Amplification Solutions immediately prior to application on the plate. It is extremely important to wash the wells properly to obtain a good test performance.

1. Determine the number of microwell strips required.
2. Wash microwell strips twice with Wash Buffer.

3. Standard dilution on the microwell plate: Add 100 µL Sample Diluent, in duplicate, to all standard wells. Pipette 100 µL prepared standard into the first wells and create standard dilutions by transferring 100 µL from well to well. Discard 100 µL from the last wells.

   Alternatively external standard dilution in tubes (see "External standard dilution" on page 3): Pipette 100 µL of these standard dilutions in the microwell strips.
4. Add 100 µL Sample Diluent in duplicate, to the blank wells.
5. Add 50 µL Sample Diluent to sample wells.
6. Add 50 µL sample in duplicate, to designated sample wells.
7. Prepare Biotin-Conjugate.
8. Add 50 µL Biotin-Conjugate to all wells.
9. Cover microwell strips and incubate 2 hours at room temperature (18° to 25°C). (Shaking is absolutely necessary for an optimal test performance.)
10. Prepare Streptavidin-HRP.
11. Empty and wash microwell strips 6 times with Wash Buffer.
12. Add 100 µL diluted Streptavidin-HRP to all wells.
13. Cover microwell strips and incubate 1 hour at room temperature (18° to 25°C). (Shaking is absolutely necessary for an optimal test performance.)
14. Prepare Amplification Solution I diluted in Amplification Diluent (1x) immediately prior to application on the plate.
15. Empty and wash microwell strips 6 times with Wash Buffer.
16. Add 100 µL Amplification Solution I to all wells.
17. Cover microwell strips and incubate for exactly 15 minutes at room temperature (18° to 25°C). (Shaking is absolutely necessary for an optimal test performance.)
18. Prepare Amplification Solution II diluted in Assay buffer immediately prior to application on the plate.
19. Empty and wash microwell strips 6 times with Wash Buffer
20. Add 100 µL Amplification Solution II to all wells.
21. Cover microwell strips and incubate for exactly 30 minutes at room temperature (18° to 25°C). (Shaking is absolutely necessary for an optimal test performance.)
22. Empty and wash microwell strips 6 times with Wash Buffer.
23. Add 100 µL of TMB Substrate Solution to all wells.
24. Incubate the microwell strips for about 10-20 minutes at room temperature (18°to 25°C).
25. Add 100 µL Stop Solution to all wells.
26. Blank microwell reader and measure color intensity at 450 nm.

Note: If instructions in this protocol have been followed samples have been diluted 1:2 (50 µL sample + 50 µL Sample Diluent). Thus concentrations read from the standard curve must be multiplied by the dilution factor (x 2).

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