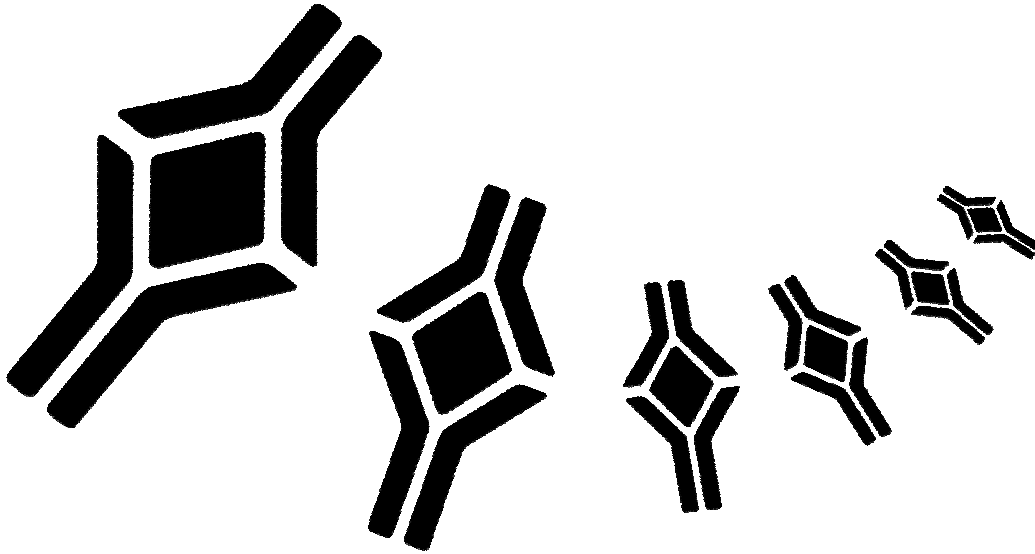


BioVendor

Research
and Diagnostic Products



PIG IFN γ ELISA

Product Data Sheet

Cat. No.: RBMS671R

For Research Use Only

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**»» This kit is manufactured by:
BioVendor – Laboratorní medicína, a.s.**

»» Use only the current version of Product Data Sheet enclosed with the kit!

1 INTENDED USE

The pig IFN γ ELISA is an enzyme-linked immunosorbent assay for quantitative detection of pig Interferon γ in cell culture supernatants, pig serum, plasma or other body fluids. **The pig IFN γ ELISA is for research use only. Not for use in diagnostic or therapeutic procedures.**

2 SUMMARY

IFN- γ , also called Type II interferon, is a homodimeric glycoprotein containing approximately 21 to 24 kD subunits.

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

IFN- γ 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, TNF- β and IFN- γ , whereas Th2 cells mainly produce IL-3, IL-4, IL-5 and IL-10, but little or no IFN- γ (5). IFN- γ preferentially inhibits the proliferation of Th2 but not Th1 cells, indicating that the presence of IFN- γ during an immune response will result in the preferential proliferation of Th1 cells (3).

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

- (1) IFN- γ is a potent activator of mononuclear phagocytes, e.g. IFN- γ stimulates the expression of Mac-1, augments endocytosis and phagocytosis by monocytes (7); and activates macrophages to kill tumor cells by releasing reactive oxygen intermediates and TNF- α (9).
- (2) IFN- γ induces or augments the expression of MHC antigens on macrophages, T and B cells and some tumor cell lines (1).
- (3) On T and B cells IFN- γ promotes differentiation. It enhances proliferation of activated B cells and can act synergistically with IL-2 to increase immunoglobulin light-chain synthesis. (4, 6). IFN- γ is one of the natural B-cell differentiation factors (8).
- (4) Finally, IFN- γ activates neutrophils, NK cells and vascular endothelial cells (2).

The role of IFN- γ as a disease marker has been demonstrated for a number of different pathological situations

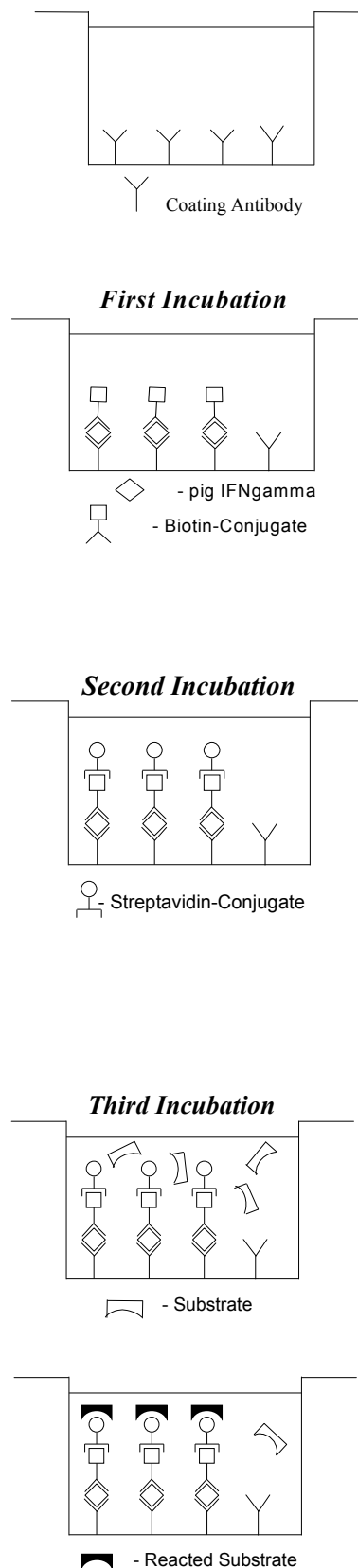
3 PRINCIPLES OF THE TEST

An anti-pig IFN γ polyclonal coating antibody is adsorbed onto microwells.

pig IFN γ present in the sample or standard binds to antibodies adsorbed to the microwells; a biotin-conjugated monoclonal anti-pig IFN γ antibody is added and binds to pig IFN γ captured by the first antibody.

Following incubation unbound biotin conjugated anti-pig IFN γ is removed during a wash step. Streptavidin-HRP is added and binds to the biotin conjugated anti-pig IFN γ . Following incubation unbound Streptavidin-HRP is removed during a wash step, and substrate solution reactive with HRP is added to the wells.

A coloured product is formed in proportion to the amount of pig IFN γ present in the sample. The reaction is terminated by addition of acid and absorbance is measured at 450nm. A standard curve is prepared from seven pig IFN γ standard dilutions and pig IFN γ sample concentration determined.



4 REAGENTS PROVIDED

- 1 aluminium poche with a **Antibody Coated Microtiter Strips** with Polyclonal Antibody (rabbit) to pig IFN γ
- 1 vial (150 μ l) **Biotin-Conjugate** anti-pig IFN γ monoclonal antibody¹⁾
- 1 vial (150 μ l) **Streptavidin-HRP¹⁾**
- 2 vials **pig IFN γ Standard**, lyophilized, 4000 pg/ml upon reconstitution
- 1 vial (5 ml) **Assay Buffer Concentrate 20x**
(PBS with 1 % Tween 20 and 10 % BSA)
- 1 bottle (50 ml) **Wash Buffer Concentrate 20x**
(PBS with 1% Tween 20)
- 1 vial (15 ml) **Substrate Solution**
- 1 vial (12 ml) **Stop Solution** (1M Phosphoric acid)
- 1 vial (0.4 ml) **Blue-Dye**
- 1 vial (0.4 ml) **Red-Dye**
- 1 vial (0.4 ml) **Green-Dye**
- 6 adhesive **Plate Covers**

Reagent Labels

¹⁾ It is recommended to spin vial in microcentrifuge before use to collect reagent at the bottom.

5 STORAGE INSTRUCTIONS

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.

The 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, the reagent is not contaminated by the first handling.

6 SPECIMEN COLLECTION

Cell culture supernatants, pig serum, plasma or other biological samples will be suitable for use in the assay. Remove serum from the clot or red cells, respectively, 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 specimens.

Clinical samples should be kept at 2° to 8°C and separated rapidly before storing at -20°C to avoid loss of bioactive pig IFN γ . If samples are to be run within 24 hours, they may be stored at 2° to 8°C. Avoid repeated freeze-thaw cycles.

For stability and suitability of samples refer to respective chapter.

7 MATERIALS REQUIRED BUT NOT PROVIDED

- 5 ml and 10 ml graduated pipettes
- 10 μ l to 1,000 μ l adjustable single channel micropipettes with disposable tips
- 50 μ l to 300 μ l 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)
- 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 linear regression analysis

8 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 statements(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 pipette 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 specimens.
- Rubber or disposable latex gloves should be worn while handling kit reagents or specimens.
- Avoid contact of substrate solution with oxidizing agents and metal.
- Avoid splashing or generation of aerosols.

- In order to avoid microbial contamination or cross-contamination of reagents or specimens which may invalidate the test use disposable pipette tips and/or pipettes.
- Use clean, dedicated reagent trays for dispensing the conjugate and substrate reagents.
- Exposure to acids will inactivate 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 specimens 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.

9 PREPARATION OF REAGENTS

A. Wash Buffer

If crystals have formed in the Wash Buffer Concentrate, warm it gently until they have completely dissolved.

Pour entire contents (50 ml) of the **Wash Buffer Concentrate** into a clean 1,000 ml graduated cylinder. Bring final volume to 1,000 ml with glass-distilled or deionized water. Mix gently to avoid foaming. The pH of the final solution should adjust to 7.4.

Transfer to a clean wash bottle and store at 2° to 25°C. Please note that the Wash Buffer is stable for 30 days. Wash Buffer may 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 |

B. Assay Buffer

Mix the contents of the bottle well. Add contents of **Assay Buffer Concentrate** (5.0 ml) to 95 ml distilled or deionized water and mix gently to avoid foaming. Store at 2° to 8°C. Please note that the Assay Buffer is stable for 30 days. Assay Buffer may 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 |

C. Preparation of Biotin-Conjugate

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

| Number of Strips | Biotin-Conjugate (ml) | Assay Buffer (ml) |
|------------------|-----------------------|-------------------|
| 1 - 6 | 0.06 | 6 |
| 1 - 12 | 0.12 | 12 |

D. Preparation of pig IFN γ Standard

Reconstitute **pig IFN γ Standard** by addition of distilled water. Refer to the Certificate of Analysis for current volume of Distilled water needed for reconstitution of standard. Mix gently to ensure complete solubilization. Store reconstituted Standard promptly at -20°C . Discard after one week.

E. Preparation of Streptavidin-HRP

Make a 1:100 dilution of the concentrated **Streptavidin-HRP** solution in **Assay Buffer** as needed according to the following table:

| Number of Strips | Streptavidin-HRP (ml) | Assay Buffer (ml) |
|------------------|-----------------------|-------------------|
| 1 - 6 | 0.06 | 6 |
| 1 - 12 | 0.12 | 12 |

F. Addition of Colour-giving Dyes

This procedure is optional, does not in any way interfere with the test results, and is designed to help the customer with the performance of the test, but can also be omitted, just following the instruction booklet.

Alternatively, the dye solutions from the stocks provided (**Blue-Dye**, **Green-Dye**, **Red-Dye**) can be added to the reagents according to the following guidelines:

1. Diluent: Before sample dilution add the **Blue-Dye** at a dilution of 1:250 (see table below) to the appropriate diluent (1x) according to the test protocol. After addition of **Blue-Dye**, proceed according to the instruction booklet.

| | |
|--------------------|-----------------------|
| 5 ml Assay Buffer | 20 µl Blue-Dye |
| 12 ml Assay Buffer | 48 µl Blue-Dye |

2. Biotin-Conjugate: Before dilution of the concentrated conjugate, add the **Green-Dye** at a dilution of 1:100 (see table below) to the Assay Buffer (1x) used for the final conjugate dilution. Proceed after addition of **Green-Dye** according to the instruction booklet, preparation of Biotin-conjugate.

| | |
|-------------------|------------------------|
| 3 ml Assay Buffer | 30 µl Green-Dye |
| 6 ml Assay Buffer | 60 µl Green-Dye |

3. Streptavidin-HRP: Before dilution of the concentrated Streptavidin-HRP, add the **Red-Dye** at a dilution of 1:250 (see table below) to the Assay Buffer (1x) used for the final Streptavidin-HRP dilution. Proceed after addition of **Red-Dye** according to the instruction booklet, preparation of Streptavidin-HRP.

| | |
|--------------------|----------------------|
| 6 ml Assay Buffer | 24 µl Red-Dye |
| 12 ml Assay Buffer | 48 µl Red-Dye |

10 TEST PROTOCOL

- a. Prepare reagents immediately before use and mix them thoroughly without foaming.
- b. 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 coated with polyclonal antibody** (rabbit) to pig IFN γ from holder and store in foil bag with the desiccant provided at 2°-8°C sealed tightly.
- c. Wash the microwell strips twice with approximately 300 μ l **Wash Buffer** per well with thorough aspiration of microwell contents between washes. Take care not to scratch the surface of the microwells.
After the last wash, empty wells and tap microwell strips on absorbent pad or paper towel to remove excess Wash Buffer. Use the microwell strips immediately after washing or place upside down on a wet absorbent paper for not longer than 15 minutes. Do not allow wells to dry.
- d. Add 100 μ l of **Assay Buffer** in duplicate to all standard wells. Prepare standard dilutions by pipetting 100 μ l of reconstituted (Refer to preparation of reagents) **pig IFN γ Standard**, in duplicate, into wells A1 and A2. Mix the contents of wells A1 and A2 by repeated aspiration and ejection, and transfer 100 μ l to well B1 and B2, respectively. Take care not to scratch the inner surface of the microwells. Continue this procedure five times, creating two rows of pig IFN γ standard dilutions ranging from 2000 to 31,25 pg/ml. Discard 100 μ l of the contents from the last microwells (G1, G2) used.

- e. Add 100 µl of **Assay Buffer**, in duplicate, to the blank wells.
- f. Add 50 µl of **Assay Buffer** to the sample wells.
- g. Add 50 µl of each **Sample**, in duplicate, to the designated wells.
- h. Cover with a **Plate Cover** and incubate at room temperature (18° to 25°C) for 1 hour on a microplate shaker set at 200 rpm.
- i. Remove Plate Cover and empty wells. Wash microwell strips 3 times according to point c. of the test protocol. Proceed immediately to the next step.
- j. Prepare **Biotin-Conjugate** (Refer to preparation of reagents).
- k. Add 100 µl of diluted **Biotin-Conjugate** to all wells, including the blank wells.
- l. Cover with a **Plate Cover** and incubate at room temperature (18° to 25°C) for 1 hour on a microplate shaker set at 200 rpm.
- m. Remove Plate Cover and empty wells. Wash microwell strips 3 times according to point c. of the test protocol. Proceed immediately to the next step.
- n. Prepare **Streptavidin-HRP** (Refer to preparation of reagents).
- o. Add 100 µl of diluted **Streptavidin-HRP** to all wells, including the blank wells.
- p. Cover with a **Plate Cover** and incubate at room temperature (18° to 25°C) for 30 minutes on a microplate shaker at 200 rpm.
- q. Remove Plate Cover and empty wells. Wash microwell strips 3 times according to point c. of the test protocol. Proceed immediately to the next step.
- r. Pipette 100 µl of **TMB Substrate Solution** to all wells, including the blank wells.

s. Incubate the microwell strips at room temperature (18° to 25°C) for about 10 minutes. Avoid direct exposure to intense light.

The colour development on the plate should be monitored and the substrate reaction stopped (see point t. of this protocol) before positive wells are no longer properly recordable.

It is recommended to add the stop solution when the highest standard has developed a dark blue colour.

Alternatively the colour development can be monitored by the ELISA reader at 620 nm. The substrate reaction should be stopped as soon as an OD of 0.6 – 0.65 is reached.

t. Stop the enzyme reaction by quickly pipetting 100 µl of **Stop Solution** into each well, including the blank wells. 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.

u. 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 pig IFN γ 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.

11 CALCULATION OF RESULTS

- Calculate the average absorbance values for each set of duplicate standards and samples. Duplicates should be within 20 per cent of the mean.
- Create a standard curve by plotting the mean absorbance for each standard concentration on the ordinate against the pig IFN γ concentration on the abscissa. Draw a best fit curve through the points of the graph.
- To determine the concentration of circulating pig IFN γ 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 pig IFN γ concentration.

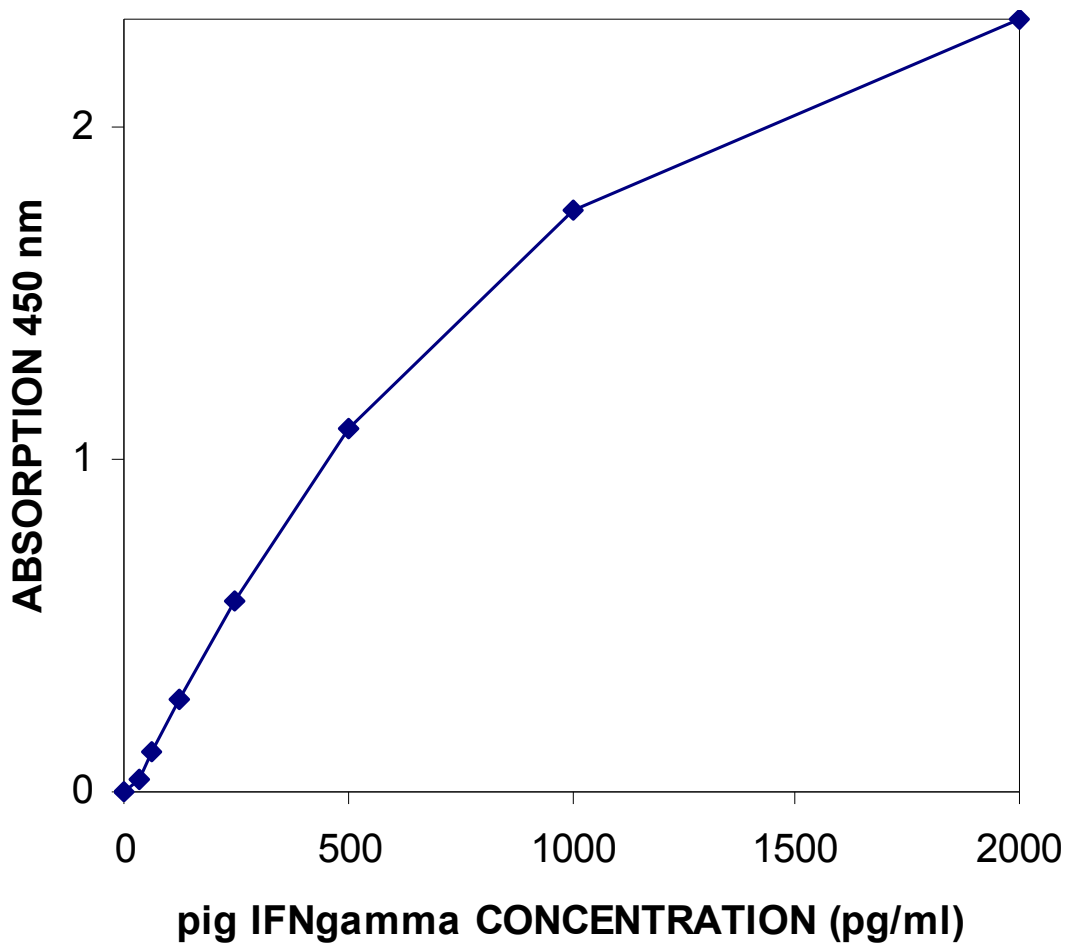
If samples have been diluted according to the instructions given in this manual (e.g. cell culture supernatants), the concentration read from the standard curve must be multiplied by the respective dilution factor.

Note: Calculation of samples with an O.D. exceeding 2.0 may result in incorrect, low pig IFN γ levels. Such samples should be re-analyzed at higher dilution rate in order to precisely quantitate the actual pig IFN γ level.

It is suggested that each testing facility establishes a control sample of known pig IFN γ concentration and runs this additional control with each assay. If the values obtained are not within the expected range of this control, the assay results may be invalid.

- A representative standard curve is shown in Figure 3. This curve cannot be used to derive test results. Every laboratory must prepare a standard curve for each group of microwell strips assayed.

Figure 3. Representative standard curve for pig IFN γ ELISA. pig IFN γ was diluted in serial two-fold steps in Sample Diluent, symbols represent the mean of three parallel titrations. Do not use this standard curve to derive test results. A standard curve must be run for each group of microwell strips assayed.



Typical data using the pig IFN γ ELISA

Measuring wavelength: 450 nm

Reference wavelength: 620 nm

| Standard | pig IFN γ Concentration (pg/ml) | O.D. (450 nm) | O.D. Mean | C.V. (%) |
|----------|--|------------------|--------------|-------------|
| 1 | 2000 | 2.387 | 2.372 | 1.9 |
| | 2000 | 2.452 | | |
| 2 | 1000 | 1.860 | 1.794 | 1.4 |
| | 1000 | 1.823 | | |
| 3 | 500 | 1.198 | 1.145 | 0.7 |
| | 500 | 1.187 | | |
| 4 | 250 | 0.655 | 0.620 | 2.6 |
| | 250 | 0.680 | | |
| 5 | 125 | 0.377 | 0.324 | 2.1 |
| | 125 | 0.366 | | |
| 6 | 62.5 | 0.217 | 0.164 | 3.3 |
| | 62.5 | 0.207 | | |
| 7 | 31.3 | 0.135 | 0.088 | 0.5 |
| | 31.3 | 0.136 | | |
| Blank | | 0.050 | 0.048 | |
| | | 0.046 | | |

The OD values of the standard curve may vary according to the conditions of assay performance (e.g. temperature effects). Furthermore shelf life of the kit may effect enzymatic activity and thus colour intensity. Values measured are still valid.

12 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 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. Completely empty wells before dispensing fresh Wash Buffer, fill with Wash Buffer as indicated for each wash cycle and do not allow wells to sit uncovered or dry for extended periods.

13 PERFORMANCE CHARACTERISTICS

A. Sensitivity

The limit of detection of pig IFN γ defined as the analyte concentration resulting in an absorption significantly higher than that of the dilution medium (mean plus two standard deviations) was determined to be 3.3 pg/ml (mean of 6 independent assays).

B. Reproducibility

a. Intra-assay

Reproducibility within the assay was evaluated in independent experiments. The overall intra-assay coefficient of variation has been calculated to be 8.4%.

b. Inter-assay

Assay to assay reproducibility within one laboratory was evaluated in independent experiments by three technicians. The overall inter-assay coefficient of variation has been calculated to be 13%.

C. Spiking Recovery

The spiking recovery was evaluated by spiking four levels of pig IFN γ into pooled normal pig serum. Recoveries were determined in two independent experiments with 4 replicates each. Observed values showed an overall mean recovery of 73%.

D. Dilution Linearity

Pig serum spiked with different levels of pig IFN γ was assayed at three serial twofold dilutions with 4 replicates each. Experiments showed an overall mean recovery of 123%.

E. Sample Stability

a. Freeze-Thaw Stability

Aliquots of spiked serum were stored frozen at -20°C and thawed up to 5 times, and pig IFN γ levels determined. There was no significant loss of IFN γ by freezing and thawing up to 5 times.

b. Storage Stability

Aliquots of spiked serum were stored at -20°C , $2-8^{\circ}\text{C}$, room temperature (RT) and at 37°C , and the pig IFN γ level determined after 24 h. There was no significant loss of pig IFN γ immunoreactivity during storage under above conditions.

F. Specificity

The interference of circulating factors of the immune systems was evaluated by spiking these proteins at physiologically relevant concentrations into a pig IFN γ positive serum. There was no detectable cross reactivity.

G. Expected Serum Values

There are no detectable IFN γ levels found in healthy pigs. Elevated pig IFN γ levels depend on the type of immunological disorder.

14 REFERENCES

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15 REAGENT PREPARATION SUMMARY

A. Wash Buffer Add **Wash Buffer Concentrate** 20 x (50 ml) to 950 ml distilled water

| B. Assay Buffer | Number of Strips | Assay Buffer Concentrate (ml) | Distilled Water (ml) |
|------------------------|------------------|-------------------------------|----------------------|
| | 1 - 6 | 2.5 | 47.5 |
| | 1 - 12 | 5.0 | 95.0 |

C. Biotin-Conjugate Make a 1:100 dilution according to the table.

| Number of Strips | Biotin-Conjugate (ml) | Assay Buffer (ml) |
|------------------|-----------------------|-------------------|
| 1 - 6 | 0.06 | 6 |
| 1 - 12 | 0.12 | 12 |

D. Standard Reconstitute **pig IFN γ Standard** by addition of distilled water. Reconstitution volume is stated on the label of the standard vial.

| E. Streptavidin-HRP | Number of Strips | Streptavidin-HRP (ml) | Assay Buffer (ml) |
|----------------------------|------------------|-----------------------|-------------------|
| | 1 - 6 | 0.06 | 6.0 |
| | 1 - 12 | 0.12 | 12.0 |

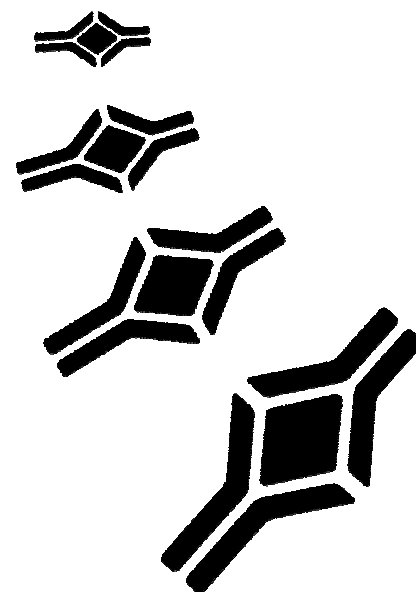
16 TEST PROTOCOL SUMMARY

- Wash microwell strips twice with **Wash Buffer**
- Add 100 µl **Assay Buffer**, in duplicate, to all standard wells
- Pipette 100 µl diluted **pig IFN γ Standard** into the first wells and create standard dilutions ranging from 2000 to 31.3 pg/ml by transferring 100 µl from well to well. Discard 100 µl from the last wells.
- Add 100 µl **Assay Buffer**, in duplicate, to the blank wells
- Add 50 µl **Assay Buffer**, in duplicate, to the sample wells
- Add 50 µl **Sample**, in duplicate, to designated wells
- Cover microwell strips and incubate **1 hour** at room temperature (18° to 25°C) on microplate shaker
- Prepare **Biotin-Conjugate**
- Empty and wash microwell strips 3 times with **Wash Buffer**
- Add 100 µl of diluted **Biotin-Conjugate** to all wells
- Cover microwell strips and incubate **1 hour** at room temperature (18° to 25°C) on microplate shaker
- Prepare **Streptavidin-HRP**
- Empty and wash microwell strips 3 times with **Wash Buffer**
- Add 100 µl of diluted **Streptavidin-HRP** to all wells
- Cover microwell strips and incubate **30 minutes** at room temperature (18° to 25°C) on microplate shaker
- Empty and wash microwell strips 3 times with **Wash Buffer**
- Add 100 µl of **TMB Substrate Solution** to all wells including blank wells
- Incubate the microwell strips for **10 minutes** at room temperature (18° to 25°C)
- Add 100 µl **Stop Solution** to all wells including blank wells
- Blank microwell reader and measure colour intensity at 450 nm

Note: Calculation of samples with an O.D. exceeding 2.0 may result in incorrect, low pig IFN γ levels. Such samples should be re-analyzed at higher dilution rate in order to precisely quantitate the actual pig IFN γ level.

NOTES





| | | | | |
|---|---|---|---|--|
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