PeliKine Compact ä
Human IFNg ELISA kit

288 tests

An enzyme immunoassay for the quantitative determination of human interferon gamma

PRODUCT INFORMATION

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Protocol summery and checklist PeliKine compact™ human IFNγ ELISA kit

Day 0:

- ○ Bring coating antibody to room temperature (18-25°C).
- ○ Prepare coating buffer.
- ○ Dilute coating antibody 1:100 in coating buffer, add 100 µl to all wells, cover the plate(s) and incubate overnight at room temperature.

Day 1:

- ○ Bring all reagents, with the exception of streptavidin-HRP, to room temperature.
- ○ Prepare blocking buffer
- ○ Wash the plate(s) five times with PBS.
- ○ Add 200 µl blocking buffer to all wells and incubate for one hour at room temperature.
- ○ Prepare standard and sample dilutions.
- ○ Prepare washing buffer.
- ○ Wash the plate(s) five times with washing buffer.
- ○ Leaving the substrate blank wells empty, add 100 µl of standard and sample dilutions to the appropriate wells, cover the plate(s) and incubate for one hour at room temperature.
- ○ Dilute biotinylated IFNγ antibody 1:100 in dilution buffer.
- ○ Wash the plate(s) five times with washing buffer.
- ○ Leaving the substrate blank wells empty, add 100 µl of the diluted biotinylated IFNγ antibody to all wells, cover the plate(s) and incubate for one hour at room temperature.
- ○ Dilute the streptavidin-HRP conjugate 1:10,000 in dilution buffer.
- ○ Wash the plate(s) five times with washing buffer.
- ○ Leaving the substrate blank wells empty, add 100 µl of the streptavidin-HRP conjugate to all wells, cover plate(s) and incubate for 30 minutes at room temperature.
- ○ Just before use, prepare substrate solution.
- ○ Wash the plate(s) five times with washing buffer.
- ○ Add 100 µl substrate solution to all wells, including the substrate blank wells, and incubate for 30 minutes at room temperature in the dark.
- ○ Add 100 µl stop solution to all wells and read the plate at 450 nm.
- ○ Calculate the amount of IFNγ in the samples.

	1	2	3	4	5	6	7	8	9	10	11	12
A	S1	S1										
B	S2	S2										
C	S3	S3										
D	S4	S4										
E	S5	S5										
F	S6	S6										
G	S7	S7										
H	S8	S8									B	B

Plate plan proposed for the Pelikine compact™ human IFNγ ELISA kit:

Key: B: substrate blank S1-S8: IFNγ standards 0 - 500 pg/ml Empty: samples

I. INTRODUCTION

At this moment fifteen interferon α (IFNα), one interferon β (IFNβ) and one interferon γ (IFNγ) have been reported. IFNγ is produced during an immune response by $CD8^+$, NK, γδ and TH1 T helper cells. It differs structurally and functionally from IFNα and IFNß; binds to distinct receptors and has pronounced immuno-regulatory effects, including activation of macrophages to enhance phagocytosis and tumour killing capability, activation and growth enhancement of cytolytic T-cells and NK-cells, and induction of class II MHC antigen and Fcγ receptor on macrophages and many other cell types.
IFNγ also regulates humeral immune responses: it induces immunoglobulin secretion by activated B-cells stimulated with IL-2 and potentiates IL-4 induced proliferation of human B-cells. IFNγ has documented antiviral and antiprotozoal activities, although IFNα and IFNß seem to have more potent antiviral activities than IFNγ
Several substances originally described for their biological activities have been identified as IFNγ; macrophage activating factor (MAF), T-cell replacing factor (TRF), Type II interferon and immune interferon.
Bioassays for the quantification of IFNγ, based on cytopatic reductive effects of IFNγ on cultured cells have been used for several years. In this assay IFNγ reduces the killing of a target cell line such as L929 (murine), HEp2C or A549 (human) cells by for example, encephalomyocarditis virus. An alternative assay system involves measurement of induction of HLA-DR antigens on tumour cells, which can be detected in a cell ELISA. These assays, although sensitive, are time consuming and might be susceptible to interference by other substances.
The Pelikine compact™ human IFNγ ELISA kit has been developed for faster, more reproducible and specific quantification of human IFNγ in serum, plasma and other body fluids, as well as in cell-culture supernatant.

II. PRINCIPLE OF THE TEST

The Pelikine compact™ human IFNγ ELISA kit is a "sandwich-type" of enzyme immunoassay in which a monoclonal anti-human IFNγ antibody is bound onto polystyrene microtiter wells. Human IFNγ, present in a measured volume of sample or standard is captured by the antibody on the microtiter plate, and non-bound material is removed by washing. Subsequently, a biotinylated polyclonal antibody to human IFNγ is added. This antibody binds to the IFNγ-antibody complex present in the microtiter well. Excess biotinylated antibody is removed by washing, followed by addition of horseradish peroxidase (HRP) conjugated streptavidin, which binds onto the biotinylated side of the IFNγ sandwich. After removal of non-bound HRP conjugate by washing, a substrate solution is added to the wells. A coloured product is formed in proportion to the amount of IFNγ present in the sample or standard. After the reaction has been terminated by the addition of a stop solution, absorbance is measured in a microtiter plate reader. From the absorbance of samples and those of a standard curve, the concentration of IFNγ can be determined by interpolation with the standard curve.

III. STORAGE AND STABILITY

The Pelikine compact™ human IFNγ ELISA kit should be stored at -18°C to -32°C. The performance of the kit is guaranteed until the expiration date shown on the case label.

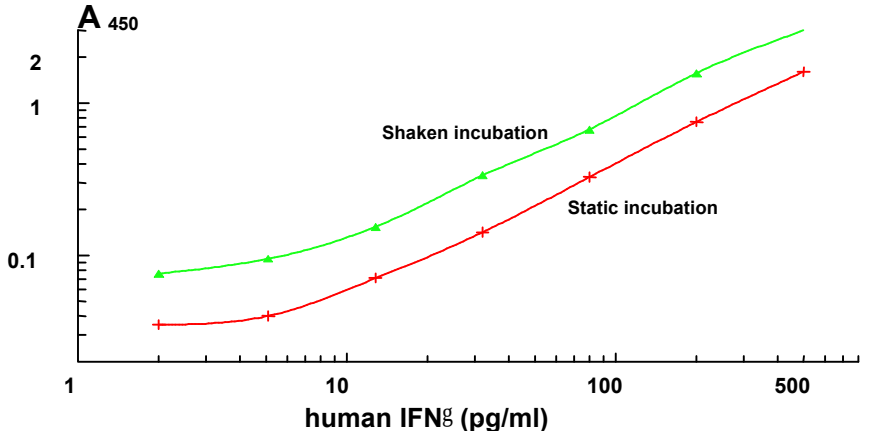
IV. CONTENTS OF THE KIT

The Pelikine compact™ human IFNγ ELISA kit contains material sufficient for 288 tests, including standard curve samples. The reagents provided are:

Quantity	Kit component		Volume	Cap colour
1 vial	coating antibody	100-fold concentrated	375 µl	red
1 vial	blocking reagent	50-fold concentrated	2 ml	transparent
2 vials	IFNγ standard (lyophilized)	4400 pg/ml	500 µl	black
1 vial	biotinylated IFNγ antibody	100-fold concentrated	375 µl	yellow
1 vial	streptavidin-HRP conjugate	10,000-fold concentrated	20 µl	brown
1 bottle	dilution buffer	5-fold concentrated	60 ml	-
3 pcs	microtiter plates + lid	-	-	-
10pcs	plate seals	-	-	-

V. PRECAUTIONS FOR USE

1) The Pelikine compact™ human IFNγ ELISA kit is intended *for research purposes only*.
2) Only use the reagents and microtiter plates supplied with the kit, do not mix reagents from different kit lots.
3) Handle all plasma and serum samples with care to prevent transmission of blood-borne infections.
4) Sodium azide inactivates HRP, do not use sodium azide-containing solutions, nor add sodium azide to the supplied materials.
5) All reagents contain thiomersal (0.001 % w/v) and may be toxic upon ingestion, inhalation or skin contact. Avoid contact of skin, eyes or clothing with dilution, washing or substrate buffer. In case of contact, wash skin or eyes with water and consult a physician.
6) The IFNγ standard contains human serum which has been found to be non-reactive for Hepatitis B surface Antigen (HBsAg), Hepatitis C Virus (HCV) and Human Immunodeficiency Virus (HIV). Nevertheless the standard should be handled as potentially hazardous and capable of transmitting diseases.
7) Centrifuge all vials before use (1 minute 3000 x g).
8) With the exception of the substrate blank wells, do not allow wells to stand uncovered or dry for extended periods between incubation steps.



Typical standard curve for the PeliKine compact™ human IFNγ ELISA kit

	STATIC INCUBATION	SHAKEN INCUBATION
	Calculated mean absorbance at 450 nm	
substrate blank	0	0
0 pg/ml	0.025	0.073
2.0 pg/ml	0.035	0.074
5.1 pg/ml	0.040	0.095
12.8 pg/ml	0.071	0.154
32 pg/ml	0.142	0.336
80 pg/ml	0.327	0.671
200 pg/ml	0.753	1.572
500 pg/ml	1.604	> 3.000

DO NOT USE THESE DATA TO CONSTRUCT A STANDARD CURVE FOR SAMPLE VALUE CALCULATIONS

Add 100 µl of stop solution to all wells.

After stopping the colour is stable for maximally 30 minutes.

15. **PLATE READ-OUT**

Read at 450 nm in an ELISA reader.

IX. RESULTS

Substrate blank

- Record the absorbance at 450 nm for the substrate blank wells and average the duplicate values.

Standard curve

- Record the absorbance at 450 nm for each well containing standard and average the duplicate values.
- Calculate the net average absorbances by subtracting the average of the substrate blank wells.
- Plot the net average absorbances (ordinate) versus the IFNγ concentration in pg/ml (abscissa) on log-linear paper and draw the best fitting curve. An example of a standard curve is given on the next page.

Samples

- Record the absorbance at 450 nm for each standard well, and average the duplicate values.
- Calculate the net average absorbances by subtracting the average of the substrate blank wells.
- Locate the net average absorbance value found for each sample on the vertical axis and follow a horizontal line intersecting the standard curve. At the point of intersection, read the IFNγ concentration (pg/ml) from the horizontal axis. Multiply the obtained IFNγ concentration with the dilution factor of the sample and record this figure.

X. INCREASED SENSITIVITY

The assay sensitivity can be increrased by a small adaption of the incubation methodology. Just follow all the instructions as stated in the assay procedure (chapter VIII), but incubate at room temperature (18-25°C) on a horizontal plate shaker at **700 ± 100 rpm**. All incubations, with exception of the enzymatic colour development, have to be completed on the shaker. This will result in an increase in assay sensitivity, with small effects on the background levels (see figure next page).

VI. ADDITIONAL BUFFERS & SOLUTIONS REQUIRED

Coating buffer: 0.1 M Carbonate/bicarbonate buffer pH 9.6

Solution A: 1.24 g $Na_2CO_3.H_2O$ in 100 ml distilled water

Solution B: 1.68 g $NaHCO_3$ in 200 ml distilled water

Take 70 ml of solution A, and add solution B until the pH is 9.6
(approximately 175 ml of solution B required)

The prepared buffer can be stored up to one week at 2-8°C.

PBS stock solution [20 x]: 0.2 M Phosphate Buffered Saline (PBS)

Dissolve 32 g $Na_2HPO_4.2H_2O$
6 g $NaH_2PO_4.2H_2O$
164 g NaCl
in 900 ml distilled water
(intensive stirring and some heating will speed dissolution).

Bring the temperature of the solution back to room temperature (18-25°C) and check pH; if necessary adjust pH to 6.8 - 6.9 with concentrated HCl or NaOH, and add distilled water to a volume of 1 liter (when diluted 20 times the obtained buffer will have a pH of 7.2 - 7.4).

Add 20 mg thiomersal as preservative. Do not use sodium azide (NaN_3) since this preservative reduces the quality of the enzymatic label.

The prepared buffer can be stored up to three months at 2-8°C.

Note: in the concentrated buffer salt crystals may appear when stored at 2-8°C. Before preparing the working-strength buffer, first warm the concentrated buffer BRIEFLY to 37°C to dissolve the precipitate.

Washing buffer: PBS with 0.005 % TWEEN 20

Make 1 liter of working-strength PBS by diluting the PBS stock solution (see above) 20-fold with distilled water.
Add 50 µl TWEEN 20.
The prepared buffer can be stored up to one month at 2-8°C.

Substrate buffer: 0.11 M acetate buffer pH 5.5

Dissolve 15.0 g sodium-acetate ($CH_3COONa.3H_2O$) in 800 ml distilled water.

Adjust pH to 5.5 with glacial acetic acid, add distilled water to a volume of 1 liter.

Do not add any preservative (e.g. merthiolate, sodium azide) since this may affect the quality of the enzymatic colour development.
The prepared buffer can be stored up to two weeks at 2-8°C

3,5,3',5'-tetramethylbenzidine (TMB) stock solution: 6 mg/ml TMB in DMSO

Dissolve 30 mg 3,5,3',5'-tetramethylbenzidine (TMB) in 5 ml dimethylsulfoxide (DMSO).
The prepared stock solution can be stored up to 1 month **at room temperature (18-25°C)** and if **protected against light**.

Hydrogen peroxide stock solution: 3% H_2O_2 solution in distilled water.

The prepared stock solution can be stored up to one month at 2-8°C.

SUBSTRATE SOLUTION

For each plate mix the following reagents:

12	ml	substrate buffer
200	µl	TMB stock solution
12	µl	H_2O_2 stock solution

The substrate solution should be prepared just before use and has to be at room temperature (18-25°C) for optimal reproducible results.

Stop solution: 1.8 M H_2SO_4 solution in distilled water.

VII. ADDITIONAL INFORMATION

Additional materials required

- Pipetting devices for accurate delivery of 1-10 µl, 50 µl, 100 µl and 1 ml volumes.
- Beakers, flasks, cylinders necessary for preparation of reagents.
- Device for delivery of washing buffer (wash bottle / automated plate washer).
- Microtiter plate reader.

The kit contains one brown capped vial of concentrated streptavidin-poly-HRP conjugate, which must be stored at –18°C to –32°C to maintain maximal stability. The contents of the vial will not be frozen at this temperature.

Per microtiter plate, add 3 µl streptavidin-poly-HRP conjugate to 30 ml of working-strength kit buffer just before use. **Do not prepare in advance of assay.**

Leaving the substrate blank wells empty, add 100 µl of streptavidin-poly-HRP conjugate to all wells.

Cover the microtiter plate(s) with adhesive seal, gently agitate the microtiter plate by tapping the edge of the frame for a few seconds to mix contents of each well and **incubate for 30 minutes at room temperature (18-25°C).**

12. **FOURTH WASH STEP**

Aspirate supernatant from wells and wash the microtiter plate(s) as decribed in point 6.

13. **FOURTH INCUBATION STEP** **Enzymatic colour development**

Approximately 10 minutes before use, prepare the substrate solution as decribed on page 4 of this leaflet.

The substrate solution should be at room temperature (18-25°C) for optimal reproducible results.

Add 100 µl of substrate solution to all wells, **including the substrate blank wells.**

Cover mictrotiter plate(s) with lid, gently agitate the microtiter plate by tapping the edge of the frame for a few seconds to mix contents of each well and **incubate for 30 minutes at room temperature (18-25°C) in the dark.**
do not cover the plate with aluminium foil.

Note: The speed of enzymatic coulour development is influenced by many factors including temperature and quality of the used TMB and H_2O_2.

14. **STOP ENZYMATIC REACTION**

Prepare washing buffer as described on page 3 of this leaflet.

Wash the required microtiter plates five times with washing buffer in a plate washer.
In case of manual washing, completely fill the wells (> 300 µl) with washing buffer and aspirate, repeat this four times. After the final aspiration the wells should be dry.

7. **FIRST INCUBATION STEP** **Standards and samples**

Leaving the substrate blank wells empty, transfer 100 µl of the prepared standards, control and samples in duplicate into the appropriate wells (see recommended plate plan).
Cover plate(s) with adhesive seal, gently agitate the microtiter plate by tapping the edge of the plate for a few seconds to mix contents of each well and **incubate for 1 hour at room temperature (18-25°C)**.

8. **SECOND WASH STEP**

Aspirate supernatant from wells and wash the microtiter plate(s) as described in point 6 above.

9. **SECOND INCUBATION STEP** **biotinylated antibody**

The kit contains one yellow-capped vial with concentrated antibody-biotin conjugate.

Per microtiter plate, add 120 µl biotinylated antibody to 12 ml working-strength kit buffer just before use.
Leaving the substrate blank wells empty, add 100 µl of diluted biotinylated antibody to all wells.
Cover plate(s) with adhesive seal, gently agitate the microtiter plate by tapping the edge of the plate for a few seconds to mix contents of each well and **incubate for 1 hour at room temperature (18-25°C)**.

10. **THIRD WASH STEP**

Aspirate supernatant from wells and wash the microtiter plate(s) as decribed in point 6.

11. **THIRD INCUBATION STEP** **Streptavidin-poly-HRP conjugate**

Sensitivity

MEAN calculated zero signal + 3 SD : 1-2 pg/ml (shake - static incubation)
2 x (MEAN calculated zero signal) : 4-6 pg/ml (shake - static incubation)

Note: the sensitivity is dependent of the type and quality of enzymatic substrate.

Expected values

IFNγ values in fresh serum and plasma samples of healthy individuals are below 10 pg/ml.

Specificity

No crossreactivity was observed with the following recombinant human proteins: IL-1α, IL-1β, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-11, Macrophage Colony Stimulating Factor (M-CSF), Granulocyte Colony Stimulating Factor (G-CSF), Granulocyte/Macrophage Colony Stimulating Factor (GM-CSF), Leukaemia Inhibitory Factor (LIF), RANTES, Stem Cell Factor/ Mast Cell Factor (SCF/MCF), Transforming Growth Factor β-1 (TGFβ-1), Tumour Necrosis Factor α (TNFα) and Tumour Necrosis Factor β (TNFβ/Lymphotoxin).

REFERENCES

Adolf,G.R. (1985) Oncology 42: 33
Aggerwal,B.B. Gutterman,J.U. (1992) Human cytokines. Blackwell Sci.Pub. ISBN 0-86542-183-8
Balkwill,F. (1989) The Lancet (I): 1060
Billiau,A. (1988) Immunology Today 9: 37
Billiau,A. *et al* (1990) Biochem.Pharmacol. 40: 1433
Bruserud,O. *et al* (1993) Eur.J.Hematol. 51: 73
Celada,A. *et al* (1989) Eur.J.Immunol. 19: 1103
Doldi,K. *et al* (1985) J.Interferon Res. 5: 55
Farrar,M.A. *et al* (1993) Ann.Rev.Immunol. 11: 571
Gray,P.W. *et al* (1892) Nature 295: 503
Grossberg, S.E. *et al* (1989) Experientia 45: 508
Ijzermans,J.M. *et al* (1989) Immunobiol. 179: 456
Kwiatkowski,D.A. *et al* (1990) The Lancet 336: 1201
Locksley, R.M. *et al* (1991) Immunoparasitology Today: A58
Mogensen, S.C. *et al* (1987) Interferon 8: 55
O'Garra,A. (1989) The Lancet (I): 1003
Paliard,X. (1988) *et al* J.Immunol. 141: 849
Reiter,Z. (1993) J.Interferon Res. 13: 247
Samuel, C.E. (1991) Virology 183: 1
Thomson,A.W. (1991) The cytokine handbook. Academic Press ISBN 0-12-689660-7

VIII. ASSAY PROCEDURE

1. *BRING ALL REAGENTS TO ROOM TEMPERATURE (18-25°C)*, with the exception of the

streptavidin-HRP conjugate, positive control and standard which has to be kept at -18°C to -32°C to ensure stability. Centrifuge all vials before use (1 minute 3000 x g).
For your convenience an easy-reference manual with check list and plate plan are available on the last pages of this leaflet.

2. DILUTION BUFFER

The kit contains one bottle with 5-fold concentrated dilution buffer.
For optimal assay results, dilute samples and standards in working-strenght dilution buffer.

Calculate the quantity of dilution buffer required (approximately 15 ml undiluted buffer per microtiter plate) and prepare a working-strength solution by diluting the opalescent concentrated buffer 5 times in distilled water before use. Shake gently. The working-strength dilution buffer can be stored for up to one week at 2-8°C.

3. MICROTITER PLATES — Coating antibody

Coating
The kit contains three microtiter plates for 96 tests each, including the standard curve and control samples.

Prepare coating buffer as described on page 3 of the information leaflet.
Per microtiter plate, add 120 µl of coating antibody (red-capped vial) to 12 ml coating buffer.
Add 100 µl to all wells, cover microtiter plate(s) with lid and **incubate overnight at room temperature (18-25°C)**.

Washing procedure

Prepare washing buffer as described on page 3 of the information leaflet.
Aspirate supernatants from wells and completely fill the wells (> 300 µl) with working-strength PBS/TWEEN and aspirate. Repeat this four times, after the final aspiration the wells should be dry.

Blocking procedure
The kit contains one transparent-capped vial with 2 ml blocking reagent.

Prepare blocking buffer by adding 500 µl blocking reagent to 25 ml working-strength PBS (1:20 dilution of stock PBS as decribed on page 3 of the information leaflet).
Add 200 µl blocking buffer to all wells, cover microtiter plate(s) with adhesive seal, gently agitate the microtiter plate by tapping the edge of the plate for a few seconds to mix contents of each well and **incubate for 1 hour at room temperature (18-25°C)**.

4. IFNg STANDARD — Standard curve preparation

A natural human IFNγ standard has been calibrated against the WHO reference preparation

(IFNγ 88/606; National Institute for Biological Standards and Control, Potters Bar, Hertfordshire, U.K. 1 WHO Unit = 53 pg IFNγ).
The kit contains two lyophilized black-capped vials with 4400 pg/ml natural IFNγ.

Reconstitute one lyophilized standard by adding 500 µl of distilled water to the vial. Incubate for 10 minutes at room temperature and mix gently. After reconstitution the standard must be kept cold (2-8°C) and stored frozen after use (<-18°C, preferably <-70°C).

Label 7 tubes, one tube for each dilution: 500, 200, 80, 32, 12.8, 5.1, and 2.0 pg/ml. Pipette 585 µl of working-strength dilution buffer into the tube labelled 500 pg/ml and 300 µl of working-strenght dilution buffer into the other tubes. Transfer 75 µl of the IFNγ standard (4400 pg/ml) into the first tube labelled 500 pg/ml, mix well and transfer 200 µl of this dilution into the second tube labelled 200 pg/ml. Repeat the serial dilutions six more times by adding 200 µl of the previous tube of diluted standard to the 300 µl of dilution buffer.
The standard curve will contain 500, 200, 80, 32, 12.8, 5.1, 2.0 and 0 pg/ml (dilution buffer).

It is recommended to prepare two separate series for each assay.

Avoid repeated freeze-thawing of the standard, although experimental data have shown that up to 3 freeze-thaw cycles have no effect on the IFNγ levels of the standard. Thaw the reconstituted standard in tap water (18-25°C), do not use 37°C or 56°C water baths for this purpose. The second vial of standard can be used in case of prolonged storage of the reconstituted standard (> 2 months).

5. SAMPLES

Serum, EDTA-anti-coagulated plasmas, and culture fluids are suitable for use in the assay (**caution**: separate plasma/serum and blood cells within 4 hours after collection, non-separated samples must be kept on temperatures from 2 to 8°C). Do not use grossly haemolyzed or lipemic specimens. If samples are to be run within 24 hours, they may be stored at 2-8°C; otherwise samples should be stored frozen (<-18°C, preferably <-70°C). Avoid freezing and thawing samples more than once. Prior to the assay, frozen samples should be thawed **as quickly as possible** in tap water (18-25°C), do not use 37°C or 56°C water baths for this purpose.

It is recommended to dilute the test samples at least 1:2 in working-strength dilution buffer. If high levels of IFNγ (> 500 pg/ml) are expected in the test samples, additional dilutions of sample i.e. 1:10 and 1:50 should also be prepared.

6. FIRST WASH STEP