Sanquin

PeliKine™ human IFNγ ELISA kit

96 tests

An enzyme immunoassay
for the quantitative determination of human Interferon gamma

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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 IFNβ; 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. 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 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 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

At arrival the Pelikine human IFNγ ELISA kit will be stable for 3 months when kept at 2-8°C. Stability until the expiration date shown on the box label can be achieved by storing the IFNγ standard, the biotynilated IFNγ antibody conjugate, and the streptavidine-HRP conjugate separately at -18 to -32°C.

IV. CONTENTS OF THE KIT

The PeliKine™ human IFNγ ELISA kit contains material sufficient for 96 tests, including standard curve samples. The reagents provided are:

Quantity	Kit component		Volume	Cap colour
1 pieces	Precoated microtiterplate	12 x 8 strips + plate-frame	-	
2 vial	IFNγ standard (lyophilized)	4400 pg/ml	500 µl**	black
1 vial	biotinylated antibody*	100-fold concentrated	200 µl	yellow
1 vial	streptavidin-poly-HRP conjugate*	10,000-fold concentrated	20 µl	brown
1 bottle	Wash buffer*	20-fold concentrated	50 ml	
1 bottle	HPE dilution buffer*	5- fold concentrated	55 ml	
1 bottle	TMB substrate solution	Ready-for-use	12.5 ml	brown
1 botle	Stop solution (0.18 M H_2SO_4)	Ready-for-use	13.5 ml	white
5 pcs	plate seals	-	-	

* These reagent contains Merthiolate (0.001% w/v) as a preservative.
** Volume after reconstitution of lyophilised material.

V. PRECAUTIONS FOR USE

1) The PeliKine™ 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) Centrifuge all vials before use (1 minute 3000 x g).
6) With the exception of the substrate blank wells, do not allow wells to stand uncovered or dry for extended periods between incubation steps.

VI. 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 wash buffer (wash bottle / automated plate washer).
– Microtiter plate reader.

Sensitivity

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

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
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

VII. ASSAY PROCEDURE

- *BRING ALL REAGENT TO ROOM REMPERATURE (18-25°C),* with the exception of the streptavidin-HRP conjugate which has to be kept at -18°C to -32°C to ensure stability. Centrifugate all vials before use (1 minute 3000 x g).
- It is advised to test all samples and standard dilutions in duplicate.
- Mix all reagents thoroughly before use (without foaming).
- **For your convenience an easy-reference manual with check list and plate plan are available on the last pages of this leaflet.**

1. **MICROTITER PLATE** **Precoated strips**
   The kit contains one plate-frame with 12 strips of 8 wells, vacuum-sealed and packed in a resealable pouch. The PeliKine™ human IFNγ ELISA kit provides the flexibility to run two partial plates on separate occasions.

Before opening the plastic pouch, determine the number of strips required to test the desired number of samples plus 18 wells needed for running standards and blanks in duplicate. Remove non-used strips from the plate-frame and restore them in the plastic pouch containing the desiccant for up to 1 month at 2-8°C

2. **BUFFER PREPARATIONS**
   Wash buffer
   The kit contains one bottle with 20 fold concentrated wash buffer.

Prepare the wash buffer by adding 50 ml of the washbuffer concentrate (total content of the bottle) to 950 ml distilled water. The diluted washbuffer can be stored for up to 2 months at 2-8°C.

Note: The concentrated buffer may contain salt crystals. Before preparing the working-strength buffer, warm the concentrated buffer BRIEFLY to 37°C to dissolve the crystals.

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

Calculate the quantity of HPE-dilution buffer required (approximately 2 ml concentrated HPE buffer per microwell strip). Prepare a working-strength solution by diluting the opalescent concentrated buffer 5 fold in distilled water. The diluted buffer can be stored for up to one week at 2-8°C.

3. IFN γ **standard** **Standard curve preparation**

A natural human IFNγ standard has been calibrated against the WHO International Standard (IFNγ 87/606; National Institute for Biological Standards and Control, Potters Bar, Hertfordshire, U.K. 1 WHO Unit = 53 pg IFNγ).
The kit contains two lyophilized vials with 4400 pg/ml natural human IFNγ.

Reconstitute one vial by adding 500 µl of distillled water. Allow 10 minutes at room temperature to dissolve and mix gently. The reconstituted standard can be stored for two hours at 2-8°C and for 1 week between -18 to -32°C.
The second vial of lyophilized standard can be used in later assays.

Label 8 tubes, one tube for each dilution: 450, 180, 72, 28.8, 11.5, 4.6, 1.8. and 0 pg/ml.
Pipette 395 µl of working-strength dilution buffer into the tube labeled 450 pg/ml and 300 µl of working-strength dilution buffer into the other tubes.
Transfer 45 µl of the IFNγ standard (4400 pg/ml) into the first tube labeled 450 pg/ml, mix well and transfer 200 µl of this dilution into the second tube labeled 180 pg/ml.
Repeat the serial dilution's 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 450, 180, 72, 28.8, 11.5, 4.6, 1.8 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.

4. SAMPLES

Serum, heparin or EDTA-anti-coagulated plasmas, and culture fluids are suitable for use in the assay. 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). Up to 3 freeze-thaw cycles have no effect on the IFNγ levels of serum or plasma samples. Nonetheless, excessive freeze-thaw cycles should be avoided. Prior to the assay, frozen samples should be thawed as quickly as possible in a 37°C water bath and then brought to room temperature (18-25°C)..

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.

5. **FIRST WASH STEP**

Prepare wash buffer as described on page 4 of this leaflet.

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

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

Leaving the substrate blank wells empty, transfer 100 µl of the prepared standards 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)**.

Just before washing, prepare next incubation reagent as described in point 8

7. **SECOND WASH STEP**

Aspirate supernatant from wells and wash the microtiter plate as described in point 5 above.

8. **SECOND INCUBATION STEP** **biotinylated antibody**

The kit contains one yellow-capped vial with biotinylated antibody.

Calculate the required amount of conjugate (10 µl per strip) and dilute 1:100 in HPE-dilution buffer.

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)**.

Just before washing prepare next incubation reagents as described in point 10.

9. **THIRD WASH STEP**

Aspirate supernatant from wells and wash the microtiter plate as described in point 5 above.

10. **THIRD INCUBATION STEP** **Streptavidin-HRP conjugate**

The kit contains one brown vial of concentrated streptavidin-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-HRP conjugate to 30 ml of working-strength dilution buffer just before use. **Do not prepare in advance of assay**.

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

Cover the microtiter plate(s) with adhesive seal, 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)**.

11. **FOURTH WASH STEP**

Aspirate supernatant from wells and wash the microtiter plate as described in point 5 above.

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

The kit contains one brown-capped bottle with a ready-for-use TMB substrate solution containing a mixture of 3,3',5,5'-tetramethylbenzidine (TMB) and hydrogen peroxide. Take care not to contaminate the TMB substrate reagent; if the solution is blue prior to use the reagent cannot be used any more.
Protect from prolonged exposure to light.
This solution must be on room temperature before use!

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

Cover microtiter 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 aluminum foil.

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

13. STOP ENZYMATIC REACTION

The kit contains one white-capped bottle with a ready-for-use stop solution of 0.18 M H2SO4.

Add 100 µl of stop solution to all wells.

After stopping the colour is stable for maximally 30 minutes.

14. PLATE READ-OUT

Read at 450 nm in an ELISA reader.

VIII. RESULTS

Substrate blank

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

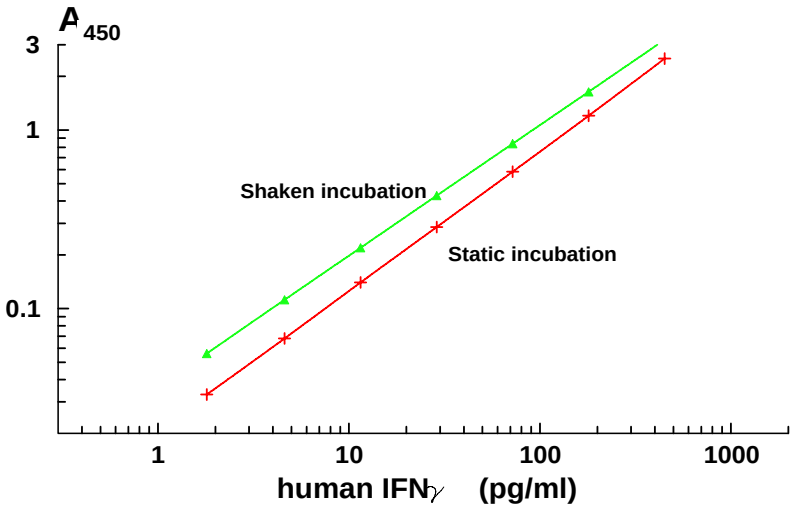
Standard curve

- 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.

A computer program to calculate ELISA results (developed by Mr E.J. Nieuwenhuys, CLB Amsterdam) is available free of charge to our kit users.
This program can be downloaded from internet:
http://www.xs4all.nl/~ednieuw/Calibration/Logit/logit.htm



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	**0.020**	**0.038**
1.8 pg/ml	**0.033**	**0.056**
4.6 pg/ml	**0.068**	**0.112**
11.5 pg/ml	**0.140**	**0.219**
28.8 pg/ml	**0.268**	**0.429**
72 pg/ml	**0.584**	**0.838**
180 pg/ml	**1.202**	**1.636**
450 pg/ml	**2.510**	**> 3.000**

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

Plate plan proposed for the PeliKine™ human IFNγ ELISA kit:

	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

Key: B: substrate blank S1-S8: IFNγ standards 0-450 pg/ml Empty: samples

Protocol summery and checklist PeliKine™ human IFNγ ELISA kit

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