Human IFNγ ELISA

DIACLONE

For research use only

950 000 096 **1 x 96 tests**
950 000 192 **2 x 96 tests**

INTENDED USE

The Human IFNγ ELISA is to be used for the in-vitro quantitative determination of interferon gamma (IFNγ) in human serum, plasma, buffered solutions or cell culture medium. The assay will recognize both natural and recombinant human IFNγ . **IFNγ ELISA is for research use only.**

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 (11).

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, TNFα and IFNγ, whereas Th2 cells main produce IL-3, IL-4, IL-5, and IL-10, but little or no IFNγ (9). 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 (7).

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 (15), and activates macrophages to kill tumor cells by releasing reactive oxygen intermediates and TNFα (21).
2/ IFNγ induces or augments the expression of MHC antigens on macrophages , T and B cells and some tumor cell lines (3).
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. (8,13). IFNγ is one of the natural B-cell differentiation factors (17).
4/ Finally, IFNγ activates neutrophils, NK cells and vascular endothelial cells (6).

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

- infections: IFNγ is produced during viral infections (4).
IFNγ is a diagnostic tool for distinguishing tuberculous from other non-tuberculous ascites (5,18).
IFNγ values in pleural fluid are significantly higher in tuberculous pleuritis patients than those in non- tuberculous pleuritis patients, with a sensitivity and a specificity of 100% (1, 2).
Therapy-induced (treatment with thalidomide) alleviation of clinical symptoms of erythema nodosum leprosum correlates with IFNγ and TNFα levels (14).
Tuberculoid leprosy patients show increased lymphocyte proliferation and IFNγ production in response to stimulation with Mycobacterium leprae as compared to lepromatous leprosy patients and healthy individuals (16).

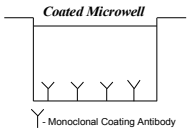
- autoimmune diseases: Accurate measurements of cellular production of cytokines, e.g. IFNγ is important in the design and monitoring of immunotherapy of multiple sclerosis (12).

- transplant rejection: Intragraft IFNγ mRNA expression occurs in active acute transplant rejection preceding clinical transplant rejection, thus offering an early diagnostic tool for detection of transplant rejection (10).

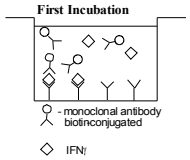
- allergy: IFNγ production by isolated lymphocytes is not detectable in patients with cow's milk allergy as compared to control individuals (19).
Infants who develop atopy produce significantly less IFNγ at birth compared to infants who do not develop atopy (20).

- diabetes: Peripheral blood lymphomononuclear cells from newly diagnosed type I diabetes produce significantly less IFNγ in comparison to controls and long standing diabetes (4).

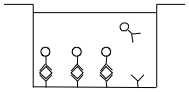
PRINCIPLES OF THE TEST



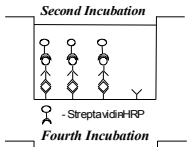
A monoclonal antibody specific for IFNγ has been coated onto the wells of the microtiter strips provided.



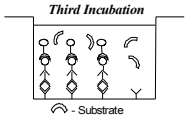
During the first incubation, IFNγ present in the sample or standard and a monoclonal anti IFNγ antibody conjugated to biotin are simultaneaously incubated.



Following incubation unbound biotinylated anti-IFNγ is removed during a wash step.



Streptavidin-HRP is added and binds to the biotinylated anti IFNγ. After incubation and a wash step a substrate solution reactive with HRP is added to the wells.



A coloured product is formed in proportion to the amount of IFNγ present in the sample. The reaction is terminated by addition of acid and absorbance is measured at 450 nm.

REAGENTS PROVIDED

REAGENTS (store at 2-8°C)	COLOUR CODE	QUANTITY 1X96 Tests 950 000 096	QUANTITY 2X96 Tests 950 000 192	RECONSTITUTION
96-wells precoated microtiter plate		1	2	Ready-to-use
Plate covers		2	4	
IFNγ Standard: 400 pg/ml	Yellow	2 vials	4 vials	Reconstitute with the volume of standard diluent indicated on the vial (see reagent preparation on page 5)
Control	Silver	2 vials	4 vials	Reconstitute with the volume of standard diluent indicated on the vial (see reagent preparation on page 5)
Standard Diluent Buffer	Black	1 bottle	1 bottle	(25 ml) 10X concentrate. Dilute in distilled water
Standard Diluent : human serum	Black	1 bottle	2 bottles	(7 ml) ready to use
Biotinylated anti IFNγ	Red	1 vial	2 vials	(0.4 ml) Dilute in biotinylated antibody diluent
Biotinylated Antibody Diluent	Red	1 bottle (7 ml)	1 bottle (13 ml)	Ready-to-use
Streptavidin-HRP		2 vials	4 vials	(5 µl) Add 0.5 ml of HRP-Diluent before further dilutions
HRP Diluent	Red	1 bottle	1 bottle	(23 ml) Ready to use
Washing Buffer	White	1 bottle	2 bottles	(10 ml) 200X concentrate. Dilute in distilled water
Substrate Solution ; chromogen TMB		1 bottle (11 ml)	1 bottle (24 ml)	Ready-to-use
Stop Reagent H_2SO_4	Black	1 bottle	2 bottles	(11 ml) Ready-to-use

MATERIALS REQUIRED BUT NOT PROVIDED

- 5 ml and 10 ml graduated pipettes
- 5 µl to 1,000 µl adjustable single channel micropipettes with disposable tips
- 50 µl to 300 µl adjustable multichannel micropipette with disposable tips
- Multichannel micropipette reservoir
- Vortex, Mixer
- 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

SAFETY

- Reagents are intended for in vitro use and are not for use in therapeutic procedures.
- All chemicals in this kit should be considered as potentially hazardous. We therefore recommend that this product is handled only by persons who have been trained in laboratory techniques and that it is used in accordance with the principles of good laboratory practice.
- The human serum included in this kit have been tested and found non reactive for HbsAg, anti HIV1 & 2 and anti VHC. Nevertheless, no known method can offer complete assurance that human blood derivatives will not transmitted hepatitis, AIDS or other infections. Therefore handling of reagents, serum or plasma specimens should be in accordance with local safety 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.
- Thoroughly mix the reagents and samples before use by agitation or swirling.
- Reagents containing preservative may be toxic if ingested.
- Avoid contact of substrate solutions with oxidizing agents and metal.

- When pipetting reagents, maintain a consistent order of addition from well-to-well. This will ensure equal incubation times for all wells.
- Respect incubation times described in the assay procedure.

- 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 a clean plastic container to prepare the washing solution.
- All residual washing liquid must be drained from the wells by efficient aspiration or by decantation followed by tapping the plate forcefully on absorbent paper. Never invert the absorbent paper directly into the wells.
- 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 solutions 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.

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 box front 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.

SPECIMEN COLLECTION, PROCESSING AND STORAGE

Cell culture supernatants, human 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.

Cell culture supernatants: Remove particulates and aggregates by spinning at approximately 1000 x g for 10 min.
Serum: Avoid any inintentional stimulation of the cells by the procedure. Use pyrogen/endotoxin free collecting tubes. Serum should be removed rapidly and carefully from the red cells after clotting. For that, after clotting, centrifuge at approximately 1000 x g for 10 min and remove serum.
Plasma: EDTA, citrate and heparin plasma can be assayed. Spin samples at 1000 x g for 30 min to remove particulates. Harvest plasma.
Storage: If not analyzed shortly after collection, samples should be aliquoted (250-500µl) to avoid freeze-thaw cycles and stored frozen at –70°C. Avoid multiple freeze-thaw cycles of frozen specimens.

Recommendation: Do not thaw by heating at 37°C or 56°C. Thaw at room temperature and make sure that sample is completely thawed and homogeneous before assaying

Samples containing a visible precipitate must be clarified prior to use in the assay. Do not use grossly hemolyzed or lipemic specimens.

For sample stability, please refer to Performance Characteristics, Sample stability.

PREPARATION OF REAGENTS

1. Washing Buffer

Dilute the **Washing Buffer Concentrate** (200X) in a clean graduated cylinder. Mix gently to avoid foaming.

Pour entire contents (10 ml) of the Washing Buffer Concentrate into a clean 2,000 ml graduated cylinder. Bring final volume to 2,000 ml with glass-distilled or deionized water. Mix gently to avoid foaming.

Transfer to a clean wash bottle and store at 2° to 25°C. Washing Buffer may be prepared as needed according to the following table:

Number of Strips	Washing Buffer Concentrate (ml)	Distilled Water (ml)
1 - 6	5	995
1 - 12	10	1,990

2. Preparation of Standard Diluent Buffer

Add the content of the vial (10X) to 225 ml distilled water before use.

3. Preparation of IFNγ Standards

Depending on the type of samples you are assaying, the kit includes two standard diluents. Because biologicals fluids might contain proteases or cytokine-binding proteins that could modify the recognition of the cytokine you want to measure. You should reconstitute standard vials with the most appropriate Standard Diluent.
For serum and plasma samples: use Standard Diluent : human serum
For cells culture supernatants: use Standard Diluent Buffer

Reconstitute IFNγ Standard by addition of appropriate Standard Diluent. Reconstitute volume is stated on the label of the standard vial.
This reconstitution produces a stock solution of 400 pg/ml IFNγ.Serial dilutions of standard must be made before each assays and cannot be stored.

4. Preparation of Controls

Freeze-dried control vials should also be reconstituted with the most appropriate Standard Diluent to your samples.
For serum and plasma samples: use Standard Diluent : human serum
For cells culture supernatants: use Standard Diluent Buffer.

Control have to be reconstituted with the volume of Standard Diluent indicated on the vial. Reconstitution of the freeze-dried material with the indicated volume, will give a solution for which the IFNγ concentration is stated on the vial. Do not store after use.

5. Preparation of biotinylated anti IFNγ

Preparation immediately before use is recommended. Dilute the biotinylated anti-IFNγ with the biotinylated antibody diluent in a clean glass vial. Biotinylated anti IFNγ may be prepared as needed according the following table. Extemporaneous preparations are recommended.

Number of Strips	Biotinylated Antibody Concentrate (µl)	Biotinylated Antibody Diluent (µl)
2	40	1,060
3	60	1,590
4	80	2,120
6	120	3,180
12	240	6,360

6. Preparation of Streptavidin-HR

It is recommended to centrifuge vial for a few seconds in a microcentrifuge to collect all the volume at the bottom

Dilute the Streptavidin-HRP 1:100 just prior to use by adding 0.5 ml of HRP diluent to the vial containing Streptavidin-HRP concentrate. DO NOT KEEP THIS DILUTION FOR FURTHER EXPERIMENTS.
Make a further dilution with HRP-Diluent in a clean glass vial as needed according to the following table:

Number of Strips	Pre-diluted Streptavidin-HRP (µl)	HRP Diluent (ml)
2	30	2
3	45	3
4	60	4
6	75	5
12	150	10

TEST PROTOCOL

a. Mix all reagents thoroughly without foaming before use.

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 control samples should be assayed in duplicate. Remove sufficient **Microwell Strips coated with Antibody to human IFN** from the aluminium pouch immediately prior to use. Return any wells not required for this assay with desiccant to the pouch. Seal tightly.

c. Add 100µl of of **appropriate Standard Diluent** (see preparation of reagents) to standard wells B1, B2, C1, C2, D1, D2, E1, E2, F1, F2.
Reconstitute **standard vial** with the appropriate volume as described in the chapter preparation of reagents. Pipette 200 µl of standard into wells A1 and A2 (see Figure 1 and 2). Transfer 100 µl from A1 and A2 to B1 and B2 wells. Mix the contents by repeated aspirations and ejections. Take care not to scratch the inner surface of microwells. Repeat this procedure from the wells B1, B2 to wells C1, C2 and from wells C1, C2 to D1, D2 and so on creating two parallel rows of IFN standard dilutions ranging from 400 to 12.5 pg/ml. Discard 100 µl from the content of the last microwells used (F1, F2).
Alternatively these dilutions can be done in separate tube and diluted standard pipetted directly into wells.

Figure 1. Preparation of IFNγ standard dilutions:

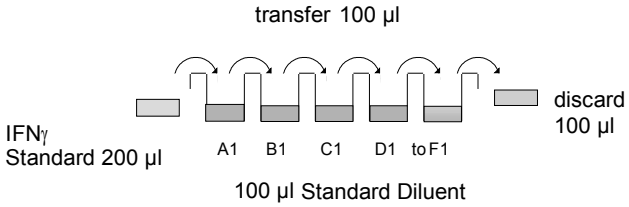


Figure 2. Diagram depicting an example of the arrangement of blanks, standards, samples and controls in the microwell strips:

	Standard Concentrations pg/mL		Sample wells									
	1	2	3	4	5	6	7	8	9	10	11	12
A	400	400										
B	200	200										
C	100	100										
D	50	50										
E	25	25										
F	12.5	12.5										
G	Blank	Blank										
H	Ctrl	Ctrl										

d. Add 100 µl of **appropriate Standard Diluent** in duplicate, to the blank wells (G1, G2).

e. Add 100 µl of **Sample** to sample wells, in duplicate, to the designated wells and 100 µl of **reconstituted control vial,** in duplicate, to control wells (H1, H2).

f. Prepare biotinylated anti IFN□: (refer to Preparation of reagents 5.).

g. Add 50 µl of **diluted biotinylated anti IFN**□ to all wells.

h. Cover with a Plate Cover and incubate at room temperature (18° to 25°C) for 2 hours.

i. Remove the cover and wash the plate as follows:
   1. Aspirate the liquid from each well;
   2. Dispense 0.3 ml of washing solution into each well;
   3. Aspirate again the content of each well;
   4. Repeat step 2. and 3. two times

j. Prepare Streptavidin-HRP solution just before use: (refer to Preparation of reagents 6.)

k. Distribute 100 µl of **Streptavidin-HRP** solution to all wells, including blanks.

l. Cover the plate and incubate the plate at room temperature (18□C to 25□C) for 30 min.

m. Remove the cover and empty wells. Wash microwell strips according to step i. Proceed immediately to the next step.

n. Pipette 100 µl of ready-to-use **TMB Substrate Solution** to all wells, including the blank wells and incubate in the dark for about 15-20 minutes at room temperature. Avoid direct exposure to light by wrapping the plate in aluminium foil.

o. Incubation time of the substrate solution is usually determined by the ELISA reader performances. Many ELISA readers record absorbance only up to 2.0 O.D. **Therefore the colour development within individual microwells must be watched by the person running the assay, and the substrate reaction stopped before positive wells are no longer properly recordable.**

p. The enzyme-substrate reaction is stopped by quickly pipetting 100 µl of **H_2SO_4 : Stop Reagent** into each well, including the blank wells to completely and uniformly inactivate the enzyme. Results must be read immediately after the H_2SO_4 : Stop Reagent is added.

q. 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, controls and the IFN□ standards.

CALCULATION OF RESULTS

- Calculate the average absorbance values for each set of duplicate standards, samples and controls. Duplicates should be within 20 per cent of the mean.

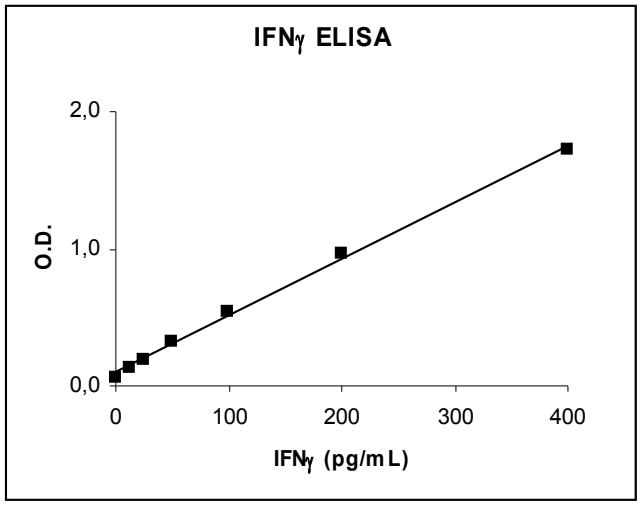
- Create a linear standard curve by plotting the mean absorbance for each standard concentration on the ordinate against the IFNγ standard concentration on the abscissa.

- To determine the concentration of IFNγ in each sample, first find the mean OD 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 IFNγ concentration.

Note: Do not extrapolate the standard curve beyond the 400 pg/ml standard curve point. The dose-response is non-linear in this region and good accuracy is difficult to obtain. Concentrated samples (> 400 pg/ml) have to be diluted with standard diluent or with your own sample buffer. During analysis, multiply results by the appropriate dilution factor.

- 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 IFNγ ELISA ranging from 12.5 to 400 pg/ml.
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 IFNγ ELISA

Measuring wavelength: 450 nm
Reference wavelength: 620 nm

Standard	IFNγ Concentration (pg/ml)	O.D. (450 nm)	O.D. Mean	C.V. (%)
1	400 400	1.71 1.73	1.72	1.0
2	200 200	0.91 1.01	0.96	7.0
3	100 100	0.53 0.55	0.54	1.8
4	50 50	0.31 0.33	0.32	3.3
5	25 25	0.19 0.19	0.19	1.9
6	12.5 12.5	0.13 0.13	0.13	0.0
Blank	0 0	0.06 0.06	0.06	-

LIMITATIONS OF THE PROCEDURE

– 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 Washing Buffer, fill with Washing Buffer as indicated for each wash cycle and do not allow wells to sit uncovered or dry for extended periods.

PERFORMANCE CHARACTERISTICS

Sensitivity

The limit of detection for IFNγ, defined as the analyte concentration resulting in an absorption significantly higher than the absorption of the dilution medium (mean plus three standard deviations) was determined to be less than 5 pg/ml (one session, 40 blank duplicates).

Reproductibility

a. Intra-assay

Reproductibility within the assay was evaluated in three independent experiments by two different technicians . Each assay was carried out with 6 replicates (3 duplicates) of 3 spiked serum or human pooled serum samples and 2 supernatants containing different concentrations of IFNγ. Data below show the mean IFNγ concentration and the coefficient of variation for each sample. **The overall intra-assay coefficient of variation has been calculated to be 4.9%.**

Session	Samples	Assay 1 [IFNγ] pg/ml	Assay 2 [IFNγ] pg/ml	Assay 3 [IFNγ] pg/ml	Mean	SD	CV
1	1	282	267	271	273	7.8	2.9
	2	144	151	149	148	3.5	2.3
	3	62	68	69	66	3.6	5.5
	4	68	62	55	62	6.9	11.1
	5	177	180	185	181	4.4	2.5
2	1	275	263	278	272	7.7	2.8
	2	150	146	150	149	2.1	1.4
	3	56	63	69	63	6.6	10.6
	4	83	77	71	77	5.9	7.7
	5	196	185	166	183	15.3	8.4
3	1	262	242	257	254	10.1	4.0
	2	127	132	130	130	2.3	1.8
	3	53	48	56	52	3.8	7.2
	4	65	63	66	65	1.7	2.6
	5	165	164	160	163	2.9	1.8

Example operator A

b. Inter-assay

Assay to assay reproductibility within one laboratory was evaluated in three independent experiments by thwo technicians. Each assay was carried out with 6 replicates of 3 spiked human pooled serum samples and 2 supernatants containing different concentration of IFNγ. Data below show the mean IFNγ concentration and the coefficient of variation.**The calculated overall coefficient of variation was 10%.**

Technician	Session	Sample 1 [IFNγ] pg/ml	Sample 2 [IFNγ] pg/ml	Sample 3 [IFNγ] pg/ml	Sample 4 [IFNγ] pg/ml	Sample 5 [IFNγ] pg/ml
A	1	282	144	62	68	177
		267	151	68	62	180
		271	149	69	55	185
	2	275	150	56	83	196
		263	146	63	77	185
		278	150	69	71	166
	3	262	127	53	65	165
		242	132	48	63	164
		257	130	56	66	160
B	1	270	142	44	82	191
		251	139	46	76	182
		261	136	51	73	180
	2	266	115	54	92	207
		245	125	62	94	208
		228	117	48	86	201
	3	283	146	55	77	162
		259	139	60	73	162
		289	145	52	79	163
Mean		264	138	56	75	180
SD		16	11	8	11	16
CV		6	8	14	14	9

Spike Recovery

The spike recovery was evaluated by spiking three concentrations of recombinant IFNγ into human serum samples or pooled normal human sera. As shown below recoveries were determined in two independent experiments with 6 replicates each. **Recoveries ranged from 88% to 100% with an overall mean recovery of 94%.**

IFNγ Spike (pg/ml)	Experiment	Recovery (%) IFNγ
400	1	95
	2	100
200	1	88
	2	81
80	1	98
	2	99

Dilution Parallelism

Three serum samples and one human pooled serum (spiked) with different levels of IFNγ were analysed at different serial two fold dilutions (1:2 – 1:8) with 2 replicates each. In the table below the per cent recovery of expected values is listed. **Recoveries ranged from 87% to 126% with an overall mean recovery of 107%.**

Sample	Dilution	IFNγ concentration (pg/ml) Expected Value	Observed Value	% Recovery of Exp. Value
1	1:2	*	444	--
	1:4	222	279	126
	1:8	140	147	105
2	1:2	*	405	--
	1:4	203	240	118
	1:8	120	126	105
3	1:2	*	381	--
	1:4	191	211	111
	1:8	105	104	99
4	1:2	*	312	--
	1:4	156	167	107
	1:8	84	72	87

Sample stability

a. Freeze-Thaw Stability

Three aliquots of 2 serum samples (spiked) were stored at -20°C and thawed up 5 times, and the IFNγ levels determined. As shown in the table below, there was a little loss of IFNγ after 5 cycles of freezing and thawing (11%)

N° of Freeze Thaw cycles	IFNγ Sample 1 Concentration (pg/ml)	Recovery (%)	IFNγ Sample 2 Concentration (pg/ml)	Recovery (%)
0	146	100	295	100
1	148	101	275	93
3	146	100	273	92
5	128	87	263	89

b. Storage stability

Aliquots of 2 serum samples (spiked) were stored at -20°C, 2-8°C, room temperature (RT) and at 37°C, and the IFNγ level determined after 24 h. As shown in the table below, there was no significant loss of IFNγ immunoreactivity during storage at RT and 2-8°C and slight loss after one night at 37°C.

Storage Temperature	IFNγ Sample 1 Concentration (pg/ml)	Recovery (%)	IFNγ Sample 2 Concentration (pg/ml)	Recovery (%)
-20°C	205	100	184	100
2-8°C	196	96	209	113
RT	208	90	199	108
37°C	173	85	165	90

Specificity

The assay recognizes both natural and recombinant human IFNγ. To define the specificity of this ELISA several proteins were tested for cross reactivity. There was no cross reactivity observed for any protein tested (IL-1α,IL-1β, IL-10, IL-12, IL-4, IL-6, TNFα, IL-8, and IL-13).

Expected serum values

A panel of 40 human sera and 40 human plasma was tested for IFNγ. All were below the detection level <5 pg/ml except one sample with a concentration of 7,5 pg/ml for serum and 24 pg/ml for plasma.

Standard Calibration

This immunoassay is calibrated against the International Reference Standard NIBSC 88/606. NIBSC 88/606 is quantitated in International Units (IU), 1IU corresponding to 0.28 ng Diaclone IFNγ.

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REAGENT PREPARATION SUMMARY

1. Washing Buffer — Add **Washing Buffer Concentrate** 200 X (10 ml) to 1990 ml distilled water

2 Standard Diluent Buffer — Add **Standard Diluent Buffer Concentrate** 10 X (25 ml) to 225 ml distilled water

3. IFNγ Standard — Reconstitute **IFNγ Standard** by addition of appropriate Standard Diluent as stated on vial label.

4. Controls — Reconstitute **controls** by addition of appropriate Standard Diluent as stated on vial label.

5. Biotinylated anti IFNγ

Number of Strips	Biotinylated Antibody Concentrate (µl)	Biotinylated Antibody Diluent (µl)
2	40	1,060
3	60	1,590
4	80	2,120
6	120	3,180
12	240	6,360

6. Streptavidin-HRP

Number of Strips	Pre-Diluted Streptavidin-HRP (µl)	HRP-Diluent (ml)
2	30	2
3	45	3
4	60	4
6	75	5
12	150	10

TEST PROTOCOL SUMMARY: Total procedure length: 2h45mn

- Add 100 µl of appropriate Standard Diluent Buffer, in duplicate, to standard wells (B1 to F2)
- Pipette 200 µl reconstituted IFNγ Standard in wells A1 and A2 and create standard dilutions ranging from 400 to 12.5 pg/ml by transferring 100 µl from well to well. Discard 100 µl from the last wells.
- Add 100 µl of appropriate **Standard Diluent Buffer** , in duplicate, to the blank wells.
- Add 100 µl of **Sample**, in duplicate, to designated wells and 100 µl of reconstituted **control**, in duplicate, to control wells.
- Prepare Biotinylated anti IFNγ
- Add 50 µl of **diluted biotinylated anti IFNγ** to all wells
- Cover microwell strips and incubate 2 hours at room temperature (18-25°C)
- Empty and wash microwell strips 3 times with **Washing Buffer**
- Prepare Streptavidin-HRP
- Add 100 µl of **diluted Streptavidin-HRP** to all wells
- Incubate 30 minutes covered at room temperature (18° to 25°C).
- Empty and wash microwell strips 3 times with **Washing Buffer**
- Add 100 µl of ready-to-use **TMB solution** to all wells including blank wells.
- Incubate the microwell strips for about 12-15 minutes at room temperature (18° to 25°C) in the dark.
- Add 100 µl H_2SO_4: **Stop Solution** to all wells including blank wells.
- Measure colour intensity at 450 nm and optinally at 620 nm as the reference wave length (610 nm to 650 nm is acceptable).

Note: Calculation of samples with an O.D. exceeding the range of the standard curve may result in incorrect, low IFNγ levels. Such samples require further dilution with appropriate Standard Diluent Buffer in order to precisely quantitate the actual IFNγ level.