Human IFNγ ELISA Kit

Instructions for use

Catalogue numbers:	1x96 tests:	950.000.096
	2x96 tests:	950.000.192

For research use only

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Human IFNγ ELISA KIT

1. Intended use

The Diaclone Human IFNγ ELISA kit is a solid phase sandwich ELISA for the *in-vitro* qualitative and quantitative determination of IFNγ in supernatants, buffered solutions or serum and plasma samples. This assay will recognise both natural and recombinant human IFNγ.

This kit has been configured for research use only. Not suitable for use in therapeutic procedures.

2. Introduction

2.1. Summary

Different populations of T-cells secrete differing patterns of cytokines that ultimately lead to different immune responses. IFNγ production is a key function of Th1, CD8+ CTLs and also NK cells. It is a cytokine critical for cell mediated immunity against viral and intracellular bacterial infections and is involved in the inflammatory response following secretion via macrophage activation and stimulation of antibody secretion. IFNγ is the hallmark effector cytokine of Th1 and therefore is an excellent marker for identifying a host response to intracellular pathogens.

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 mainly produce IL-3, IL-4, IL-5, and IL-10, but little or no IFNγ (*1*). 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 (*2*).

In addition, IFNγ has several properties related to immunoregulation. IFNγ is a potent activator of mononuclear phagocytes (*3*), and activates macrophages to kill tumor cells by releasing reactive oxygen intermediates and TNFα (*4*). IFNγ induces or augments the expression of MHC antigens on macrophages, T and B cells and some tumor cell lines (*5*). 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 (*6*,*7*).

The role of IFNγ as a disease marker has been demonstrated for a number of different pathological situations including, viral infection (*8*), autoimmune disease (*9*), transplant rejection (*10*), diabetes (*5*) and allergy (*11*).

2.2. Principle of the method

A capture Antibody highly specific for IFNγ has been coated to the wells of the microtiter strip plate provided during manufacture. Binding of IFNγ samples and known standards to the capture antibodies and subsequent binding of the Biotinylated anti-IFNγ secondary antibody to the analyte is completed during the same incubation period. Any excess unbound analyte and secondary antibody is removed.
The HRP conjugate solution is then added to every well including the zero wells, following incubation excess conjugate is removed by careful washing.
A chromogen substrate is added to the wells resulting in the progressive development of a blue coloured complex with the conjugate. The colour development is then stopped by the addition of acid turning the resultant final product yellow. The intensity of the produced coloured complex is directly proportional to the concentration of IFNγ present in the samples and standards.
The absorbance of the colour complex is then measured and the generated OD values for each standard are plotted against expected concentration forming a standard curve. This standard curve can then be used to accurately determine the concentration of IFNγ in any sample tested.

3. Reagents provided and reconstitution

Reagents (Store@2-8°C)	**Quantity 1x96-well kit** Cat no. 950.000.096	**Quantity 2x96-well kit** Cat no. 950.000.192	**Reconstitution**
Anti-IFNγ Coated Plate	1	2	Ready to use (96 well strip pre-coated plate)
Plastic plate covers	2	4	n/a
IFNγ Standard: 400 pg/ml	2	4	Reconstitute as directed on the vial (see Assay preparation, section 8)
Standard Diluent (Buffer)	1 (15ml)	1 (25ml)	10x concentrate, dilute in distilled water (see Assay preparation, section 8)
Standard Diluent Serum	1 (7 ml)	2 (7 ml)	Ready to use
IFNγ Control	2	4	Reconstitute as directed on the vial (see Assay preparation, section 8)
Biotinylated Anti-IFNγ	1 (0.4ml)	2 (0.4ml)	Dilute in Biotinylated Antibody Diluent (see Assay preparation, section 8)
Biotinylated Antibody Diluent	1 (7ml)	1 (13ml)	Ready to use
Streptavidin-HRP	2 (5µl)	4 (5µl)	Add 0.5ml of Streptavidin-HRP Diluent prior to use (see Assay preparation, section 8)
Streptavidin-HRP Diluent	1 (12ml)	1 (23ml)	Ready to use
Wash Buffer	1 (10ml)	2 (10ml)	200x concentrate dilute in distilled water (see Assay preparation, section 8)
TMB Substrate	1 (11ml)	1 (24ml)	Ready to use
H_2SO_4 Stop Reagent	1 (11ml)	2 (11ml)	Ready to use

4. Materials required but not provided

- Microtiter plate reader fitted with appropriate filters (450 nm required with optional 620 nm reference filter)
- Microtiter plate washer or wash bottle
- 10, 50, 100, 200 and 1,000µl adjustable single channel micropipettes with disposable tips
- 50-300µl multi-channel micropipette with disposable tips
- Multichannel micropipette reagent reservoirs
- Distilled water
- Vortex mixer
- Miscellaneous laboratory plastic and/or glass, if possible sterile

5. Storage Instructions

Store kit reagents between 2 and 8°C. Immediately after use remaining reagents should be returned to cold storage (2-8°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.

Wash Buffer 1X: Once prepared, store at 2-8°C for up to 1 week.

Standard Diluent Buffer 1X: Once prepared, store at 2-8°C for up to 1 week.

Reconstituted Standard/Control: Once prepared use immediately and do not store.

Diluted Biotinylated Anti-IFNγ: Once prepared use immediately and do not store.

Diluted Streptavidin-HRP: Once prepared use immediately and do not store.

6. Specimen collection, processing & 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: Use pyrogen/endotoxin free collecting tubes. Serum should be removed rapidly and carefully from the red cells after clotting. Following 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 analysed shortly after collection, samples should be aliquoted (250-500µl) to avoid repeated 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 use. When possible avoid use of badly haemolysed or lipemic sera. If large amounts of particles are present these should be removed prior to use by centrifugation or filtration.

7. Safety & precautions for use

- Handling of reagents, serum or plasma specimens should be in accordance with local safety procedures, e.g. CDC/NIH Health manual: "Biosafety in Microbiological and Biomedical Laboratories" 1984.
- Laboratory gloves should be worn at all times.
- Avoid any skin contact with H_2SO_4 and TMB. In case of contact, wash thoroughly with water.
- Do not eat, drink, smoke or apply cosmetics where kit reagents are used.
- Do not pipette by mouth.
- When not in use, kit components should be stored refrigerated as indicated on vials or bottles labels.
- All reagents should be warmed to room temperature before use. Lyophilized standards should be discarded after use.
- Once the desired number of strips has been removed, immediately reseal the bag to protect the remaining strips from deterioration.
- Cover or cap all reagents when not in use.
- Do not mix or interchange reagents between different lots.
- Do not use reagents beyond the expiration date of the kit.
- Use a clean disposable plastic pipette tip for each reagent, standard, or specimen addition in order to avoid cross contamination, for the dispensing of H_2SO_4 and TMB Substrate solutions, avoid pipettes with metal parts.
- Use a clean plastic container to prepare the washing solution.
- Thoroughly mix the reagents and samples before use by agitation or swirling.
- 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 insert absorbent paper directly into the wells.
- The TMB Substrate solution is light sensitive. Avoid prolonged exposure to light. Also, avoid contact of the TMB Substrate solution with metal to prevent colour development. Warning TMB Substrate is toxic avoid direct contact with hands. Dispose off properly.
- If a dark blue colour develops within a few minutes after preparation, this indicates that the TMB solution has been contaminated and must be discarded. Read absorbances within 1 hour after completion of the assay.
- When pipetting reagents, maintain a consistent order of addition from well-to-well. This will ensure equal incubation times for all wells.
- Follow incubation times described in the assay procedure.
- Dispense the TMB Substrate within 15 min of the washing of the microtiter plate.

8. Assay Preparation

Bring all reagents to room temperature before use

8.1. Assay Design

Determine the number of microwell strips required to test the desired number of samples plus appropriate number of wells needed for running zeros and standards. Each sample, standard, zero and control should be tested **in duplicate**. Remove sufficient microwell strips for testing from the pouch immediately prior to use. Return any wells not required for this assay with desiccant to the pouch. Seal tightly and return to 2-8°C storage.

Example plate layout (example shown for a 6 point standard curve)

	Standards / Controls		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	zero	zero										
H	Ctrl	Ctrl										

All remaining empty wells can be used to test samples in duplicate

8.2. Preparation of Wash Buffer

If crystals have formed in the concentrate Wash Buffer, warm it gently until complete dissolution.

Dilute the (200X) concentrate Wash Buffer 200 fold with distilled water to give a 1X working solution. Pour entire contents (10 ml) of the concentrate Wash Buffer 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˚-25˚C.

8.3. Preparation of Standard Diluent Buffer 1X

If crystals have formed in the concentrate Standard Diluent, warm it gently until complete dissolution.

Dilute the (10X) concentrate Standard Diluent 10 fold with distilled water to give a 1X working solution. Pour entire contents of the concentrate Standard Diluent into a clean appropriate graduated cylinder. Bring to final volume with glass-distilled or deionized water. Transfer to a clean wash bottle and store at 2˚-25˚C. Please see example volumes below:

Standard Diluent concentrate (ml)	Distilled water (ml)
15	135
25	225

8.4. Preparation of Standard

Depending on the type of samples you are assaying, the kit may include two Standard Diluents. Because biological 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 - Serum
For **cell culture supernatants:** use Standard Diluent Buffer 1X

Standard vials must be reconstituted with the volume of Standard Diluent shown on the vial immediately prior to use. This reconstitution gives a stock solution of 400 pg/ml of IFNγ. Mix the reconstituted standard gently by inversion only. Serial dilutions of the standard are made directly in the assay plate to provide the concentration range from 400 to 12.5 pg/ml. A fresh standard curve should be produced for each new assay.

- Immediately after reconstitution add 200µl of the reconstituted standard to wells A1 and A2, which provides the highest concentration standard at 400 pg/ml.
- Add 100µl of Standard Diluent to the remaining standard wells B1 and B2 to F1 and F2.
- Transfer 100µl from wells A1 and A2 to B1 and B2. Mix the well contents by repeated aspirations and ejections taking care not to scratch the inner surface of the wells.
- Continue this 1:1 dilution using 100µl from wells B1 and B2 through to wells F1 and F2 providing a serial diluted standard curve ranging from 400 pg/ml to 12.5 pg/ml.
- Discard 100µl from the final wells of the standard curve (F1 and F2).

Alternatively these dilutions can be performed in separate clean tubes and immediately transferred into the relevant wells.

8.5. Preparation of Control

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 - Serum
For **cells culture supernatants** : use Standard Diluent Buffer 1X

The supplied Control must 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 at the concentration stated on the vial. Do not store after use.

8.6. Preparation of Biotinylated Anti-IFNγ

It is recommended this reagent is prepared immediately before use. Dilute the Biotinylated Anti-IFNγ with the Biotinylated Antibody Diluent in an appropriate clean glass vial using volumes appropriate to the number of required wells. Please see example volumes below:

Number of wells required	Biotinylated Antibody (µl)	Biotinylated Antibody Diluent (µl)
16	40	1060
24	60	1590
32	80	2120
48	120	3180
96	240	6360

8.7. Preparation of Streptavidin-HRP

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

Dilute the 5µl vial with 0.5ml of Streptavidin-HRP Diluent **immediately before use.** Do not keep this diluted vial for future experiments. Further dilute the HRP solution to volumes appropriate for the number of required wells in a clean glass vial. Please see example volumes below:

Number of wells required	Streptavidin-HRP (µl)	Streptavidin-HRP Diluent (ml)
16	30	2
24	45	3
32	60	4
48	75	5
96	150	10

9. Method

We strongly recommend that every vial is mixed thoroughly without foaming prior to use.

Prepare all reagents as shown in section 8.
Note: final preparation of Biotinylated Antibody (section 8.6) and Streptavidin-HRP (section 8.7) should occur immediately before use.

Assay Step		Details
1.	Addition	**Prepare standard curve** as shown in section 8.4 above and add in duplicate to appropriate wells
2.	Addition	Add 100µl of each **Sample, Control and zero (appropriate Standard Diluent)** in duplicate to appropriate number of wells
3.	Addition	Add 50µl of diluted **Biotinylated Anti-IFNγ** to all wells
4.	Incubation	Cover with a plastic plate cover and incubate at room temperature (18 to 25°C) for **2 hours**
5.	Wash	Remove the cover and wash the plate as follows: a) Aspirate the liquid from each well b) Dispense 0.3 ml of **1x Wash Buffer** into each well c) Aspirate the contents of each well d) Repeat step b and c another two times
6.	Addition	Add 100µl of diluted **Streptavidin-HRP** solution into all wells
7.	Incubation	Cover with a plastic plate cover and incubate at room temperature (18 to 25°C) for **30 min**
8.	Wash	Repeat wash step 5.
9.	Addition	Add 100µl of ready-to-use **TMB Substrate** into all wells
10.	Incubation	Incubate in the dark for **12-15 minutes*** at room temperature. Avoid direct exposure to light by wrapping the plate in aluminium foil.
11.	Addition	Add 100µl of **H_2SO_4 Stop Reagent** into all wells
Read the absorbance value of each well (immediately after step 11.) on a spectrophotometer using 450 nm as the primary wavelength and optionally 620 nm as the reference wave length (610 nm to 650 nm is acceptable).		

** Incubation time of the TMB substrate is usually determined by the ELISA reader performance. Many ELISA readers only record absorbance up to 2.0 O.D. Therefore the colour development within individual microwells must be observed by the analyst, and the substrate reaction stopped before positive wells are no longer within recordable range.*

10. Data Analysis

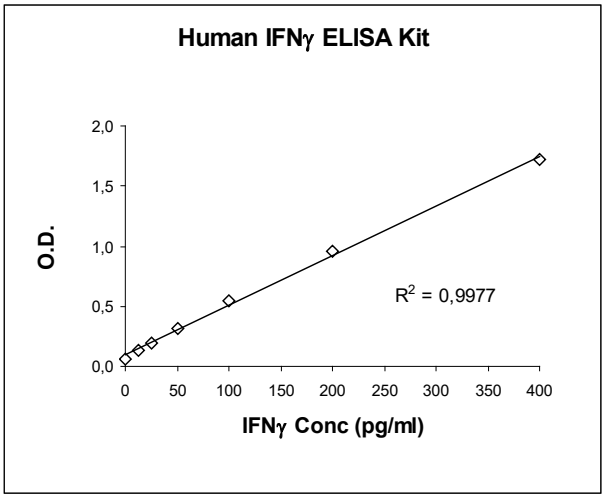
Calculate the average absorbance values for each set of duplicate standards, control and samples. Ideally duplicates should be within 20% of the mean.

Generate a linear standard curve by plotting the average absorbance of each standard on the vertical axis versus the corresponding IFNγ standard concentration on the horizontal axis.

The amount of IFNγ in each sample is determined by extrapolating OD values against IFNγ standard concentrations using the standard curve**.**

Example IFNγ Standard curve

Standard	IFNγ Conc (pg/ml)	OD (450nm) mean	CV (%)
1	400	1.720	1.03
2	200	0.958	7.02
3	100	0.541	1.83
4	50	0.319	3.33
5	25	0.190	1.87
6	12.5	0.129	0.00
zero	0	0.064	1.11



Note: curve shown above should not be used to determine results. Every laboratory must produce a standard curve for each set of microwell strips assayed.

11. Assay limitations

Do not extrapolate the standard curve beyond the maximum standard curve point. The dose-response is non-linear in this region and good accuracy is difficult to obtain. Concentrated samples above the maximum standard concentration must be diluted with Standard Diluent Buffer or with your own sample buffer to produce an OD value within the range of the standard curve. Following analysis of such samples always multiply results by the appropriate dilution factor to produce actual final concentration.

The influence of various drugs on end results has not been investigated. Bacterial or fungal contamination and laboratory cross-contamination may also cause irregular results.

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.

Disposable pipette tips, flasks or glassware are preferred, reusable glassware must be washed and thoroughly rinsed of all detergents before use.

As with most biological assays conditions may vary from assay to assay therefore **a fresh standard curve must be prepared and run for every assay.**

12. Performance Characteristics

12.1. Sensitivity

The sensitivity or minimum detectable dose of IFNγ using this Diaclone IFNγ ELISA kit was found to be 5**pg/ml**. This was determined by adding 2 standard deviations to the mean OD obtained when the zero standard was assayed in 40 times.

12.2. 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-4, IL-6, IL-8, IL-10, IL-12, IL-13, TNFα.

12.3. Precision

Intra-assay
Reproducibility within the assay was evaluated in three independent experiments. Each assay was carried out with 6 replicates (3 duplicates) of samples containing different concentrations of IFNγ: 3 in human pooled serum and 2 in supernatant. 1 standard curve was run on each plate. Data below show the mean IFNγ concentration and the coefficient of variation for each sample.
The calculated overall coefficient of variation was 4.8%.

Session	***Sample***	**Mean IFNγ** pg/ml	**SD**	**CV %**
Session 1	***Sample 1***	273.33	7.77	**2.8**
	Sample 2	148.00	3.61	**2.4**
	Sample 3	66.33	3.79	**5.7**
	Sample 4	61.67	6.51	**10.6**
	Sample 5	180.67	4.04	**2.2**
Session 2	***Sample 1***	272.00	7.94	**2.9**
	Sample 2	148.67	2.31	**1.6**
	Sample 3	62.67	6.51	**10.4**
	Sample 4	77.00	6.00	**7.8**
	Sample 5	182.33	15.18	**8.3**
Session 3	***Sample 1***	253.67	10.41	**4.1**
	Sample 2	129.67	2.52	**1.9**
	Sample 3	52.33	4.04	**7.7**
	Sample 4	64.67	1.53	**2.4**
	Sample 5	163.00	2.65	**1.6**

Inter-assay
Assay to assay reproducibility within one laboratory was evaluated in three independent experiments by two technicians. Each assay was carried out with 6 replicates (3 duplicates) of samples containing different concentrations of IFNγ: 3 in human pooled serum and 2 in supernatant. 1 standard curve was run on each plate. Data below show the mean IFNγ concentration and the coefficient of variation for each sample.
The calculated overall coefficient of variation was 10.2%.

	Sample 1	***Sample 2***	***Sample 3***	***Sample 4***	***Sample 5***
Mean IFNγ pg/ml	264	138	56	75	180
SD	16	11	8	10	16
CV%	**5.9**	**8.2**	**13.9**	**14.1**	**9.0**

12.4. 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 to 1:8) with two replicates each.
Recoveries ranged from 87 to 126% with an overall **mean recovery of 108%.**

12.5. Spike Recovery

The spike recovery was evaluated by spiking 3 concentrations of IFNγ in human serum in 2 separate experiments.
Recoveries ranged from 81 to 100% with an overall **mean recovery of 94%.**

12.6. Stability

Storage Stability
Aliquots of spiked serum and spiked medium were stored at –20°C, +2-8°C, room temperature (RT) and at 37°C and the IFNγ level determined after 24h. There was no significant loss of IFNγ reactivity during storage at +2-8°C and RT and a slight loss after 24h at 37°C (13%).

Freeze-thaw Stability
Aliquots of spiked serum and spiked medium were stored frozen at –20°C and thawed up to 5 times and the IFNγ level was determined. There was a slight loss (12%) of IFNγ after 5 cycles of freezing and thawing.

12.7. Expected serum values

A panel of 40 human sera and 40 human plasma was tested for IFNγ. All were below 5.5 pg/ml except one sample with a concentration of 7.5 pg/ml for serum and 24 pg/ml for plasma.

12.8. Standard Calibration

This immunoassay is calibrated against the International Reference Standard NIBSC 88/606.
NIBSC 88/606 is quantitated in International Units (IU) and equivalence in ng is indicated.
It has been calculated that **1IU NIBSC (approximately 50 pg) corresponding to 280 pg Diaclone IFNγ.**

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15. Assay Summary

Total procedure length: 2h45min

Add 100µl of Samples, Control and diluted Standards and 50µl diluted Biotinylated Antibody

↓

Incubate 2 hours at room temperature

↓

Wash three times

↓

Add 100µl of diluted Streptavidin-HRP

↓

Incubate 30 min at room temperature

↓

Wash three times

↓

Add 100µl of TMB Substrate
Protect from light. Let the color develop for 12-15 min.

↓

Add 100µl of Stop Reagent

↓

Read Absorbance at 450 nm

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