Human IFNγ ELISA Set

Instructions for use

Catalogue numbers:

1x96 tests:	EA101307
5x96 tests:	EA101308
10x96 tests:	EA101309
15x96 tests:	EA101310
20x96 tests:	EA101311

For research use only

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Human IFNγ ELISA Set

1. Intended use

The OriGeneIFNγ ELISA Set is intended for use in a ‘do it yourself’ solid phase sandwich ELISA for the *in-vitro* qualitative and quantitative determination of IFNγ in supernatants, buffered solutions, serum, plasma samples and other body fluids. This assay will recognise both natural and recombinant human IFNγ.

This kit has been configured for research use only.

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 noIFNγ (*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 Th1cells (*2*).

In addition, IFN-γ 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. Basic principle of a typical ELISA method

A capture Antibody highly specific for IFNγ is coated to the wells a microtitre strip plate. 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
(Details below shown for the 5x96 Set)

Reagents (Store@2-8^{o}C)	**Quantity 5x96 well kit** Cat no. EA101308	**Reconstitution**
IFNγ Standard: 400pg/ml	5 vials	Reconstitute as directed on the vial (see Assay preparation, section9)
Capture Antibody	1 vial (0.5ml)	Sterile, dilute prior to use (see Plate preparation, section8)
Biotinylated anti-IFNγ Detection Antibody	1 vial	Reconstitute with 0.55ml of reconstitution buffer prior to use (see Assay preparation, section9)
Streptavidin-HRP	1 vial (25µl)	Dilute prior to use (see Assay preparation, section9)
TMB Substrate	2 vials (25ml)	Ready to use

4. Materials required but not provided

- 96 well Microtitre plates (e.g. Nunc Maxisorp Cat # 468667)
- Reconstitution Buffer (1xPBS, 0.09% Azide)
- Coating Buffer (1xPBS, pH 7.2-7.4)
- Wash Buffer (1xPBS, 0.05% Tween20)
- Blocking Buffer (1xPBS, 5% BSA)
- Standard and Secondary Antibody Dilution Buffer (1xPBS, 1% BSA)
- HRP Diluent Buffer (1xPBS, 1% BSA, 0.1% Tween20)
- Stop Reagent (1M Sulfuric Acid)
- Microtitre plate reader with appropriate filters (450nm required with optional 620nm reference filter)
- Microplate washer or wash bottle
- 10, 50, 100, 200 and1,000µl 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 2and 8°C. Immediately after use remaining reagents should be returned to cold storage (2-8°C). Expiry of the reagents is stated on box front labels. The expiry of the components can only be guaranteed if the components are stored properly, and if, incase of repeated use of one component, the reagent is not contaminated by the first handling.

Reconstitution Buffer: Once prepared store at 2-8°Cfor up to one week
Coating Buffer: Once prepared store at 2-8°C for up to one week
Wash Buffer: Once prepared use immediately
Blocking Buffer: Once prepared store at 2-8°C for up to one week
Standard and Secondary Antibody Dilution Buffer: Once prepared store at 2-8°C for up to one week
HRP Diluent Buffer: Once prepared store at 2-8°C for up to one week

Reconstituted Biotinylated anti IFNγ Detection Antibody: Once prepared store at 2-8°C for up to one year
Reconstituted IFNγ Standard: Discard after use

6. Specimen collection, processing & storage

Cell culture supernatants, human serum, plasma or other biological samples will be suitable for use in theassay. Remove serum from the clot or red cells, respectively, as soon as possible after clotting andseparation.

Cell culture supernatants: Remove particulates and aggregates by spinning at approximately 1000 x g for 10 min.

Serum: Use pyrogen/endotoxin freecollecting 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 analyzed 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
- 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 or frozen as indicated on vials or bottles labels
- All reagents should be warmed to room temperature before use. Lyophilized standards should be discarded after use
- 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 substrate solution, 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 solution is light sensitive. Avoid prolonged exposure to light. Also, avoid contact of the TMB solution with metal to prevent colour development. Warning TMB 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 absorbance’s 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 solution within 15 min of the washing of the microtitre plate

8. Plate Preparation

8.1. Capture Antibody

For one plate add 100μl of Capture Antibody into 10mL of Coating Buffer

8.2. Preparation method

<table>
<tr><td>1.</td><td>Addition</td><td>Add 100μl of diluted Capture Antibody to every well</td></tr>
<tr><td>2.</td><td>Incubation</td><td>Cover with a plastic plate cover and incubate at 4°C overnight</td></tr>
<tr><td>3.</td><td>Wash</td><td>Remove the cover and wash the plate as follows:
a) Aspirate the liquid from each well
b) Dispense 0.4 ml of washing solution into each well
c) Aspirate the contents of each well
d) Repeat step b and c</td></tr>
<tr><td>4.</td><td>Addition</td><td>Add 250μl of Blocking Buffer to every well</td></tr>
<tr><td>5.</td><td>Incubation</td><td>Cover with a plastic plate cover and incubate at room temperature (18 to 25°C) for 2 hour(s)</td></tr>
<tr><td>6.</td><td>Wash</td><td>Remove the cover and wash the plate as follows:
a) Aspirate the liquid from each well
b) Dispense 0.4 ml of washing solution into each well
c) Aspirate the contents of each well
d) Repeat step b and c another 2 times</td></tr>
<tr><td colspan="3">For Immediate use of the plate(s) continue to section 9.

If you wish to store the coated and blocked plates for future use bench dry each plate at room temperature (18 to 25°C) for 24 hours. Cover the plates and then store at 2-8°C in a sealed plastic bag with desiccant for up to 12months.</td></tr>
</table>

9. Assay Preparation

Bring all reagents to room temperature before use

9.1. 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 and zero should be tested **in duplicate**.

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

	Standards		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												

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

9.2. Preparation of Standard

Standard vials must be reconstituted with the volume of standard dilution buffer shown on the vial immediately prior to use. This reconstitution gives a stock solution of 400pg/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 12.5pg/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 400pg/ml
- Add 100μl of appropriate 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 400pg/ml to 12.5pg/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 immediatelytransferred directly into the relevant wells.

9.3. Preparation of Biotinylated anti-IFNγ Detection Antibody

It is recommended this reagent is prepared **immediately before use**. Dilute the reconstituted biotinylated anti-IFNγ with the biotinylated antibody diluent in an appropriate clean glass vial.

For one plate add 100µl of the reconstituted detection antibody into 5mL of Biotinylated Antibody dilution buffer.

Please note for 1 x 96 tests, Biotinylated detection antibody is provided in liquid form.

9.4. 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 5µl of Streptavidin-HRP into 0.5ml of HRP diluent buffer**immediately before use.**
Take 150µl of the diluted HRP solution into 10mL of HRP diluent buffer.

Do-not keep these solutions for future experiments.
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10. Method

We strongly recommend that every vial is mixed thoroughly without foaming prior to use except the standard vialwhich must be mixed gently by inversion only.

Note: Final preparation of Biotinylated anti-IFNγ (section 9.3) and Streptavidin-HRP (section 9.4) should occur immediately before use.

Assay Step		Details
1	Preparation	**Prepare Standard curve** as shown in Section 9.2
2	Addition	Add 100µl of each **standard, sample, zero** (Standard Dilution Buffer) to appropriate wells in duplicate
3	Addition	Add 50µl of diluted **Detection Antibody** into 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 0.4 ml of **washing solution** into each well c) Aspirate the contents of each well d) Repeat step b and c
6	Addition	Add 100µl of **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 mins**
8	Wash	Repeat wash step 5.
9	Addition	Add 100µl of ready-to-use **TMB Substrate Solution** into all wells
10	Incubation	Incubate in the dark for **5-15 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 substrate solution 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*

11. Data Analysis

Calculate the average absorbance values for each set of duplicate standards 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**.**

12. 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 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 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.

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**afresh standard curve must be prepared and run for every assay.**

13. Performance Characteristics

13.1. Sensitivity

The sensitivity, minimum detectable dose of this IFNγ antibody pair was determined using the OriGene IFNγ ELISA kit (which contains the same antibodies) and was found to be **<5pg/ml**. This was determined by adding 3 standard deviations to the mean OD obtained when the zero standard was assayed 40 times.

13.2. Specificity

The assay recognizes natural human IFNγ. To define specificity of this IFNγ antibody pair, several proteins were tested for cross reactivity using the OriGene IFNγ pre-coated ELISA kit (which contains the same antibodies).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).

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