

# Human IFN- $\gamma$ ELISA Kit

For the quantitative determination of human interferon- $\gamma$  (IFN- $\gamma$ ) concentrations in serum, plasma, cell culture supernatant, and other biological fluids.

Catalogue Number: EL10024

*96 tests*

FOR LABORATORY RESEARCH USE ONLY.  
NOT FOR USE IN DIAGNOSTIC PROCEDURES.

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## INTENDED USE

This Human IFN- $\gamma$  ELISA kit is to be used for the *in vitro* quantitative determination of concentrations in serum, plasma, cell culture supernatant, and other biological fluids. This kit is intended FOR LABORATORY RESEARCH USE ONLY and is not for use in diagnostic or therapeutic procedures.

## INTRODUCTION

Interferon gamma (IFN- $\gamma$ ) is a multifunctional protein first observed to have antiviral activity in cultures of Sindbis virus-infected human leukocytes stimulated by PHA. <sup>(1)</sup> The biochemistry and biological activities of the interferons have been extensively reviewed. Produced by both CD4<sup>+</sup> and CD8<sup>+</sup> T lymphocytes and natural killer (NK) cells, IFN- $\gamma$  is now known to be both an inhibitor of viral replication and a regulator of numerous immunological functions. IFN- $\gamma$  influences the class of antibody produced by B cells up-regulates classes I and II MHC complex antigens and increases the efficiency of macrophage-mediated killing of intracellular parasites. <sup>(2, 3)</sup> Most of the activities attributed to IFN- $\gamma$  are believed to be mediated by IFN- $\gamma$ -induced proteins. The appearance of such proteins is a consequence of IFN- $\gamma$  binding to a specific receptor that is distinct from the receptor for IFN- $\alpha$  and  $\beta$ . <sup>(4)</sup> Human IFN- $\gamma$  is reported to be active only on human and non-human primate cells. <sup>(5)</sup> The biochemistry and biological activities of the interferons have been extensively reviewed. <sup>(2-9)</sup>

Human IFN- $\gamma$  is a 143 amino acid residue glycoprotein with MW of 20 or 25 kDa that demonstrates little sequence homology to IFN- $\alpha$  and  $\beta$ . <sup>(10-13)</sup> Naturally occurring IFN- $\gamma$  is found as either of two molecular-weight-species, differing in degree of glycosylation. The 25 kDa species is glycosylated at both potential N-linked glycosylation sites on the molecule (Asn 25 and 97), while the 20 kDa species is glycosylated only at Asn97. <sup>(17, 18)</sup> In neither case glycosylation is required for biological activity. <sup>(13, 16)</sup> Two allelic variants of IFN- $\gamma$  have been described differing by the presence of an Arg or Gln at position 137. <sup>(10, 16)</sup>

Although the cDNA encoding for IFN- $\gamma$  predicts a protein of 146 amino acid residues, the form secreted by mammalian cells shows a truncation of three amino acid residues from the N-terminus and the conversion of the fourth residue from glutamic acid to pyroglutamate. <sup>(11)</sup> The secreted form of IFN- $\gamma$  has no potential for the formation of disulfide bonds. <sup>(13)</sup> Human IFN- $\gamma$  apparently exists as a head-to-tail dimer in solution with the C-terminus of one monomer aligned with the N-terminus of the other monomer. <sup>(18, 19)</sup>

IFN- $\gamma$  possesses a variety of functions. Produced by CD8<sup>+</sup>, NK and TH2 T helper cells, IFN- $\gamma$  has documented antiviral, antiprotozoal and immunomodulatory activities, <sup>(20-24)</sup> although IFN- $\alpha$  and IFN- $\beta$  seem to have more potential antiviral activities than IFN- $\gamma$ . <sup>(24)</sup> The antiprotozoal activity of IFN- $\gamma$  against *Toxoplasma* and *Chlamydia* is believed to result from indoleamine 2, 3-dioxygenase activity, an enzyme induced by IFN- $\gamma$ . <sup>(25)</sup> The immunomodulatory effects of IFN- $\gamma$  are extensive and diverse. In monocyte/macrophages, IFN- $\gamma$  increases expression of class 1 MHC antigens; increases the

production of IL-1, platelet-activating factor, H<sub>2</sub>O<sub>2</sub>, and pterin; protects monocytes against LAK cell-mediated lysis; down-regulates IL-8 mRNA expression that is up-regulating TGF- $\beta$  receptor expression and up-regulating expression of the IL-2R $\gamma$  subunit. <sup>(23, 25, 26-29)</sup> It has also been demonstrated to be chemotactic for monocytes but not neutrophils. <sup>(30)</sup> IFN- $\gamma$  selectively enhances both Ig G<sub>2a</sub> secretion by LPS-stimulated B cell activation. <sup>(31, 32)</sup> IFN- $\gamma$  has also been reported to induce its own expression. IFN- $\gamma$  production accompanying local inflammation results in the induction of IFN- $\gamma$  mRNA synthesis at distant sites. This effect could be due to circulating IFN- $\gamma$  or the production of IFN- $\gamma$  by migrating cells <sup>(33)</sup>. IFN- $\gamma$  has also been shown to up-regulate ICAM-1 but not E-selectin or VCAM-1 expression on endothelial cells <sup>(34)</sup>. Finally, IFN- $\gamma$  has recently been implicated in the development of a cholinergic phenotype in embryonic septal neurons. In cultures of rat septal nuclei, IFN- $\gamma$  induced the development of cholinergic neurons

*This IFN- $\gamma$  ELISA is a 2.5 hour solid phase immunoassay readily applicable to measure IFN- $\gamma$  levels in serum, plasma, cell culture supernatant, and other biological fluids in the range of 0 to 1600 pg/mL. This IFN- $\gamma$  ELISA recognizes both natural and recombinant human IFN- $\gamma$  and exhibits no detectable cross-reactivity with human; SAA, EGF, IL-6, IL-8, IL-16, M-CSF, GM-CSF, TGF- $\beta_1$ , RANTES, FGF, MIP-1 $\alpha$ , CRP, EPO. It is expected to be effectively used for further investigations into the relationship between IFN- $\gamma$  and various diseases.*

## **PRINCIPLE OF THE ASSAY**

This IFN- $\gamma$  enzyme-linked immunosorbent assay (ELISA) applies a technique called a quantitative sandwich immunoassay. The microtiter plate provided in this kit has been pre-coated with a monoclonal specific for IFN- $\gamma$ . Standards or samples are then added to the appropriate microtiter plate wells and incubated. IFN- $\gamma$  if present, will bind and become immobilized by the antibody pre-coated on the wells. The microtiter plate wells are thoroughly washed to remove unbound IFN- $\gamma$  and other components of sample. In order to quantitatively determine the amount of IFN- $\gamma$  present in the sample, a standardized preparation of horseradish peroxidase (HRP)-conjugated polyclonal antibody specific for IFN- $\gamma$  is added to each well to "sandwich" the IFN- $\gamma$  immobilized during the first incubation. The microtiter plate then undergoes a second incubation. The wells are thoroughly washed to remove all unbound HRP-conjugated antibodies and a TMB (3,3',5, 5' tetramethyl-benzidine) substrate solution is added to each well. The enzyme (HRP) and substrate are allowed to react over a short incubation period. Only those wells that contain IFN- $\gamma$  and enzyme-conjugated antibody will exhibit a change in colour. The enzyme-substrate reaction is terminated by the addition of a sulphuric acid solution and the colour change is measured spectrophotometrically at a wavelength of 450nm  $\pm$  2nm.

In order to measure the concentration of IFN- $\gamma$  in the samples this kit contains two calibration diluents. (Calibrator Diluent I for serum/plasma testing and Calibrator Diluent II for cell culture supernatant/ urine testing). According to the testing system, the provided standard is diluted (2-fold) with the appropriate Calibrator Diluent and assayed at the same time as the samples. This allows the operator to produce a standard curve of Optical

Density (O.D.) versus IFN- $\gamma$  concentration (pg/mL). The concentration of IFN- $\gamma$  in the samples is then determined by comparing the O.D. of the samples to the standard curve.

### REAGENTS PROVIDED

All reagents provided are stored at 4°C. Refer to the expiration date on the label.

		<b>96 tests</b>
1.	<b>IFN-<math>\gamma</math> MICROTITER PLATE</b> (Part 30121) _____ Pre-coated with anti-human IFN- $\gamma$ monoclonal antibody.	<b>96 wells</b>
2.	<b>IFN-<math>\gamma</math> CONJUGATE</b> (Part 30122) _____ Anti-human IFN- $\gamma$ polyclonal antibody conjugated to horseradish peroxidase with preservative.	<b>14 mL</b>
3.	<b>IFN-<math>\gamma</math> STANDARD</b> (Part 30123) _____ Recombinant human IFN- $\gamma$ (3.2 ng/vial) in a buffered protein base with preservative, lyophilized.	<b>2 vials</b>
4.	<b>CALIBRATOR DILUENT I</b> (Part 30003) _____ Animal serum with preservative. <i>For serum/plasma testing.</i>	<b>22 mL</b>
5.	<b>CALIBRATOR DILUENT II</b> (Part EL 30004) _____ Cell culture medium with calf serum and preservative. <i>For cell culture supernatant/urine testing.</i>	<b>22 mL</b>
6.	<b>WASH BUFFER (20X)</b> (Part 30005) _____ 20-fold concentrated solution of buffered surfactant.	<b>60 mL</b>
7.	<b>SUBSTRATE A</b> (Part 30006) _____ Buffered solution with H <sub>2</sub> O <sub>2</sub> .	<b>10 mL</b>
8.	<b>SUBSTRATE B</b> (Part 30007) _____ Buffered solution with TMB.	<b>10 mL</b>
9.	<b>STOP SOLUTION</b> (Part 30008) _____ 2N Sulphuric Acid (H <sub>2</sub> SO <sub>4</sub> ). Caution: Caustic Material!	<b>14 mL</b>

## MATERIALS REQUIRED BUT NOT SUPPLIED

1. Single or multi-channel precision pipettes with disposable tips: 10-100 $\mu$ L and 50-200 $\mu$ L for running the assay.
2. Pipettes: 1 mL, 5 mL 10 mL, and 25 mL for reagent preparation.
3. Multi-channel pipette reservoir or equivalent reagent container.
4. Test tubes and racks.
5. Polypropylene tubes or containers (25 mL).
6. Erlenmeyer flasks: 100 mL, 400 mL, 1 L and 2 L.
7. Microtiter plate reader (450 nm  $\pm$  2nm)
8. Automatic microtiter plate washer or squirt bottle.
9. Sodium hypochlorite solution, 5.25% (household liquid bleach).
10. Deionized or distilled water.
11. Plastic plate cover.
12. Disposable gloves.
13. Absorbent paper.

## PRECAUTIONS

1. Do not substitute reagents from one kit lot to another. Standard, conjugate and microtiter plates are matched for optimal performance. Use only the reagents supplied by manufacturer.
2. Allow kit reagents and materials to reach room temperature (20-25°C) before use. Do not use water baths to thaw samples or reagents.
3. Do not use kit components beyond their expiration date.
4. Use only deionized or distilled water to dilute reagents.
5. Do not remove microtiter plate from the storage bag until needed. Unused strips should be stored at 2-8°C in their pouch with the desiccant provided.
6. Use fresh disposable pipette tips for each transfer to avoid contamination.
7. Do not mix acid and sodium hypochlorite solutions.
8. Human serum and plasma should be handled as potentially hazardous and capable of transmitting disease. Disposable gloves must be worn during the assay procedure since no known test method can offer complete assurance that products derived from human blood will not transmit infectious agents. Therefore, all blood derivatives should be considered potentially infectious and good laboratory practices should be followed.
9. All samples should be disposed of in a manner that will inactivate human viruses.  
Solid Wastes: Autoclave 60 min. at 121°C.  
Liquid Wastes: Add sodium hypochlorite to a final concentration of 1.0%. The waste should be allowed to stand for a minimum of 30 minutes to inactivate the virus before disposal.
10. Substrate Solution is easily contaminated. If bluish prior to use, *do not use*.
11. Substrate B contains 20% acetone, keep this reagent away from sources of heat or flame.
12. If Wash Buffer (20X) is stored at a lower temperature (2-5°C), crystals may form which must be dissolved by warming up to 37°C prior to use.

## SAMPLE PREPARATION

### 1. COLLECTION, HANDLING, AND STORAGE

- a) **Cell Culture Supernatant:** Centrifuge to remove any visible particulate material.
- b) **Serum:** Blood should be drawn using standard venipuncture techniques and serum separated from the blood cells as soon as possible. Samples should be allowed to clot for one hour at room temperature, centrifuged for 10 minutes (4°C), and serum extracted.
- c) **Plasma:** Blood should be drawn using standard venipuncture techniques and plasma collected using sodium citrate, EDTA, or heparin as an anticoagulant. To ensure optimal recovery and minimal platelet contamination, after collection there must be quick separation of plasma with less than 30 minutes on ice. Centrifuge for 10 minutes (4°C) to remove any particulates. *This IFN- $\gamma$  ELISA kit is not affected by haemolysis of specimens. No adverse effects have been noted in the presence of anti-coagulants, sodium citrate, EDTA, or heparin.*
  - Avoid grossly hemolytic, lipidic or turbid samples.
  - Serum, plasma, cell culture supernatant, and urine samples to be used within 24-48 hours may be stored at 2-8°C, otherwise samples must stored at -20°C to avoid loss of bioactivity and contamination. Avoid freeze-thaw cycles.
  - When performing the assay slowly bring samples to room temperature.
  - It is recommended that all samples be assayed in duplicate.
  - DO NOT USE HEAT-TREATED SPECIMENS.

## PREPARATION OF REAGENTS

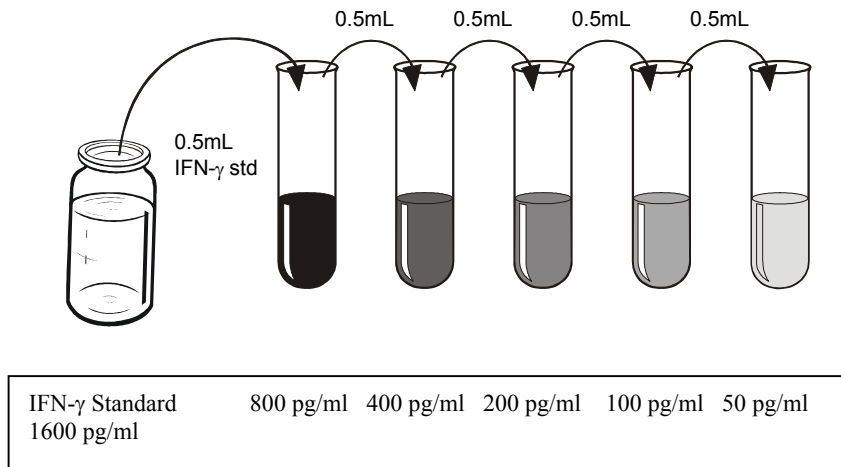
Remove all kit reagents from refrigerator and allow them to reach room temperature (20-25°C). Prepare the following reagents as indicated below. Mix thoroughly by gently swirling before pipetting. Avoid foaming.

1. **Wash Buffer (1X):** Add 60 mL of Wash Buffer (20X) and dilute to a final volume of 1200 mL with distilled or deionized water. Mix thoroughly. If a smaller volume of Wash Buffer (1X) is desired, add 1 volume of Wash Buffer (20X) to 19 volumes of distilled or deionized water. Wash Buffer (1X) is stable for 1 month at 2-8°C. Mix well before use .
2. **Substrate Solution:** Substrate A and Substrate B should be mixed together in equal volumes up to 15 minutes before use. Refer to the table below for correct amounts of Substrate Solution to prepare.

Strips Used	Substrate A (mL)	Substrate B (mL)	Substrate Solution (mL)
2 strips (16 wells)	1.5	1.5	3.0
4 strips (32 wells)	3.0	3.0	6.0
6 strips (48 wells)	4.0	4.0	8.0
8 strips (64 wells)	5.0	5.0	10.0
10 strips (80 wells)	6.0	6.0	12.0
12 strips (96 wells)	7.0	7.0	14.0

3. **IFN- $\gamma$  Standard:**

- a) Two vials of Standards are provided in this kit to allow both serum/plasma and cell culture supernatant/urine testing. Reconstitute the IFN- $\gamma$  Standard with either 2.0 mL of Calibrator Diluent I (for serum/plasma testing) or Calibrator Diluent II (for cell culture supernatant/urine testing). This reconstitution produces a stock solution of 1600 pg/mL. Allow solution to sit for at least 15 minutes with gentle agitation prior to making dilutions. Use within one hour of reconstituting. The IFN- $\gamma$  standard stock solution can be stored frozen (-20°C) for up to 30 days. Avoid freeze-thaw cycles. Aliquot if repeated use is expected.
- b) Use the above stock solution to produce a serial 2-fold dilution series within the range of this assay (50 pg/mL to 1600 pg/mL) as illustrated. Add 0.5 mL of the appropriate Calibrator Diluent to each test tube. Between each test tube transfer be sure to mix contents thoroughly. The undiluted IFN- $\gamma$  Standard will serve as the high standard (1600 pg/mL) and the Calibrator Diluent will serve as the zero standard (0 pg/mL).



## ASSAY PROCEDURE

1. Prepare Wash Buffer (1X) and IFN- $\gamma$  Standards before starting assay procedure (see Preparation of Reagents). *It is recommended that the table and diagram provided be used as a reference for adding Standards or Samples to the Microtiter Plate.*

Wells	Contents	Wells	Contents
<b>1A, 1B</b>	Standard 1 - <b>0 pg/mL</b> (S1)	<b>2A, 2B</b>	Standard 5 - <b>400 pg/mL</b> (S5)
<b>1C, 1D</b>	Standard 2 - <b>50 pg/mL</b> (S2)	<b>2C, 2D</b>	Standard 6 - <b>800 pg/mL</b> (S6)
<b>1E, 1F</b>	Standard 3 - <b>100 pg/mL</b> (S3)	<b>2E, 2F</b>	Standard 7 - <b>1600 pg/mL</b> (S7)
<b>1G, 1H</b>	Standard 4 - <b>200 pg/mL</b> (S4)	<b>2G-12H</b>	IFN- $\gamma$ samples

	1	2	3	4	5	6	7	8	9	10	11	12
<b>A</b>	S1	S5	2	6	10	14	18	22	26	30	34	38
<b>B</b>	S1	S5	2	6	10	14	18	22	26	30	34	38
<b>C</b>	S2	S6	3	7	11	15	19	23	27	31	35	39
<b>D</b>	S2	S6	3	7	11	15	19	23	27	31	35	39
<b>E</b>	S3	S7	4	8	12	16	20	24	28	32	36	40
<b>F</b>	S3	S7	4	8	12	16	20	24	28	32	36	40
<b>G</b>	S4	1	5	9	13	17	21	25	29	33	37	41
<b>H</b>	S4	1	5	9	13	17	21	25	29	33	37	41

2. Add 100  $\mu$ L of Standard or Sample to the appropriate well of the antibody pre-coated Microtiter Plate. Mix, by gently tapping the plate. Cover and incubate for 1 hour at room temperature.

3. Wash the Microtiter Plate using one of the specified methods indicated below:

Manual Washing: Remove incubation mixture by aspirating contents of the plate into a sink or proper waste container. Using a squirt bottle, fill each well completely with Wash Buffer (1X) then aspirate contents of the plate into a sink or proper waste container. Repeat this procedure four more times for a **total of FIVE washes**. After final wash, invert plate, and blot dry by hitting plate onto absorbent paper or paper towels until no moisture appears. *Note:* Hold the sides of the plate frame firmly when washing the plate to assure that all strips remain securely in frame.

Automated Washing: Aspirate all wells, then wash plates **FIVE times** using Wash Buffer (1X). Always adjust your washer to aspirate as much liquid as possible and set fill volume at 350  $\mu$ L/well/wash (range: 350-400  $\mu$ L). After final wash, invert plate, and blot dry by hitting plate onto absorbent paper or paper towels until no moisture appears. *It is recommended that the washer be set for a soaking time of 10 seconds or shaking time of 5 seconds between washes.*

4. Dispense two (2) drops or 100ul of conjugate to each well. Mix well. Cover and incubate for 1 hour at room temperature.

5. Prepare Substrate Solution no more than 15 minutes before end of second incubation (see Preparation of Reagents).
6. Repeat wash procedure as described in Step 3.
7. Add 100  $\mu$ L Substrate Solution to each well. Cover and incubate for 15 minutes at room temperature.
8. Add 100  $\mu$ L Stop Solution to each well. Mix well.
9. Read the Optical Density (O.D.) at 450 nm using a microtiter plate reader set within 30 minutes.

### **CALCULATION OF RESULTS**

The standard curve is used to determine the amount of IFN- $\gamma$  in an unknown sample. The standard curve is generated by plotting the average O.D. (450 nm) obtained for each of the standard concentrations on the vertical (Y) axis versus the corresponding IFN- $\gamma$  concentration (pg/mL) on the horizontal (X) axis.

1. First, calculate the mean O.D value for each standard and sample. All O.D. values are subtracted by the value of the zero-standard (0 pg/mL) before result interpretation. Construct the standard curve using graph paper or statistical software.
2. To determine the amount of IFN- $\gamma$  in each sample, first locate the O.D. value on the Y-axis and extend a horizontal line to the standard curve. At the point of intersection, draw a vertical line to the X-axis and read the corresponding IFN- $\gamma$  concentration. If samples generate values higher than the highest standard, dilute the samples with the appropriate Calibrator Diluent and repeat the assay, the concentration read from the standard curve must be multiplied by the dilution factor.

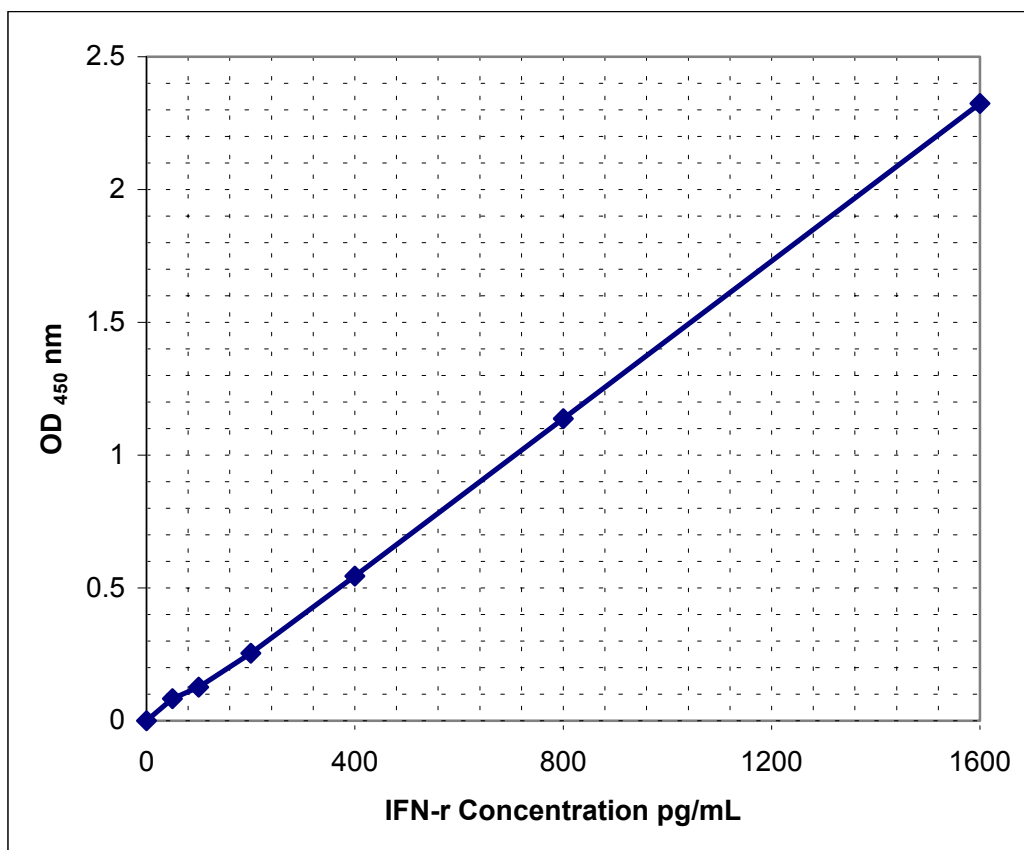
### **TYPICAL DATA**

Results of a typical standard run of a IFN- $\gamma$  ELISA are shown below. Any variation in standard diluent, operator, pipetting and washing technique, incubation time or temperature, and kit age can cause variation in result. The following examples are for the purpose of illustration only, and should not be used to calculate unknowns. Each user should obtain their own standard curve

**EXAMPLE ONE**

The following data was obtained for a standard curve using Calibrator Diluent I.

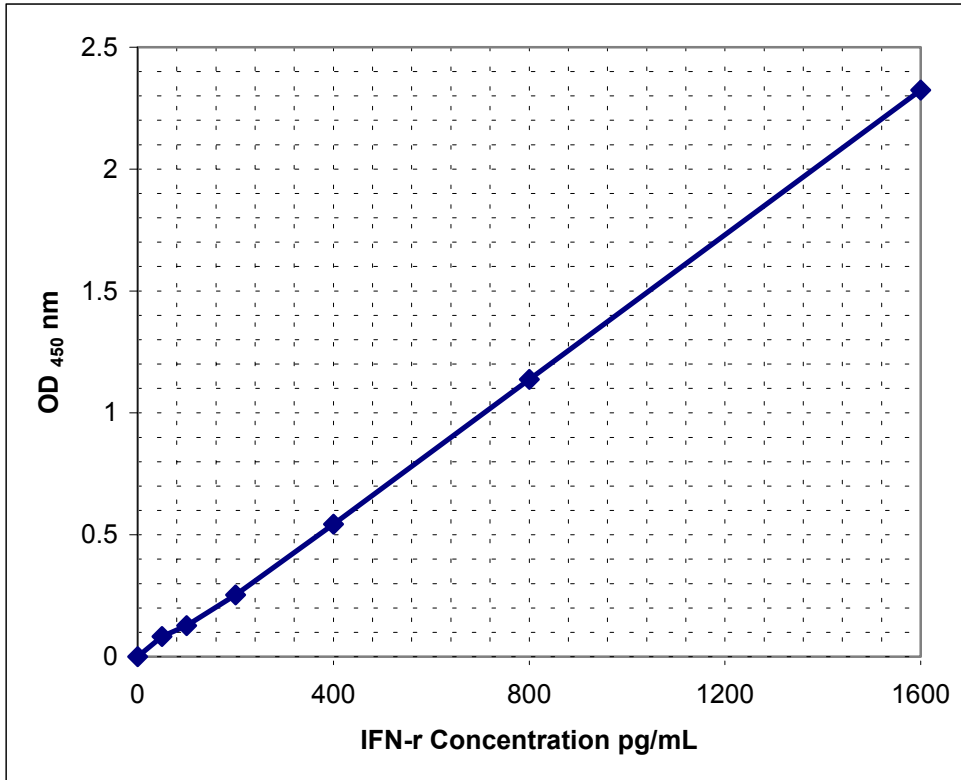
Standard (pg/mL)	O.D. (450 nm)	Mean	Zero Standard Subtracted (Std.)-(S1)
0	0.060, 0.064	0.062	0
50	0.150, 0.154	0.152	0.09
100	0.220, 0.222	0.221	0.152
200	0.360, 0.362	0.361	0.299
400	0.630, 0.634	0.632	0.570
800	1.180, 1.190	1.185	1.123
1600	2.334, 2.330	2.332	2.270



**EXAMPLE TWO**

The following data was obtained for a standard curve using Calibrator Diluent II.

Standard (pg/mL)	O.D. (450 nm)	Mean	Zero Standard Subtracted (Std.) -(S1)
0	0.070, 0.072	0.071	0
50	0.152, 0.156	0.154	0.083
100	0.196, 0.200	0.198	0.127
200	0.320, 0.330	0.325	0.254
400	0.610, 0.620	0.615	0.544
800	1.206, 1.209	1.208	1.137
1600	2.400, 2.390	2.395	2.324



## PERFORMANCE CHARACTERISTICS

### 1. INTRA-ASSAY PRECISION

To determine within-run precision, three different samples of known concentration were assayed by replicates of 20 in 1 assay.

Sample	Calibrator Diluent I assay			Calibrator Diluent II assay		
	1	2	3	1	2	3
n	20	20	20	20	20	20
Mean (pg/mL)	78.2	338.3	1208.3	75.2	318.6	1219.3
Standard Deviation (pg/mL)	10.2	33.1	75.0	5.87	22.2	59.6
Coefficient of Variation (%)	13	9.8	6.2	7.8	7.0	4.9

### 2. INTER-ASSAY PRECISION

To determine between-run precision, three different samples of known concentration were assayed by replicates on 20 different assays.

Sample	Calibrator Diluent I assay			Calibrator Diluent II assay		
	1	2	3	1	2	3
n	20	20	20	20	20	20
Mean (pg/mL)	72.3	285	1160.8	77.5	304.8	1128
Standard Deviation (pg/mL)	4.4	12.8	54.5	7.9	13.1	99.8
Coefficient of Variation (%)	6.1	4.4	4.7	10.1	43	8.8

### 3. RECOVERY

The recovery of IFN- $\gamma$  spiked to 3 different levels throughout the range of the assay in various matrices was evaluated.

Sample Type	Average Recovery %	Range %
Cell culture media	104	89 -110
Serum	102	95 -116
Plasma	100	90 - 106

### 4. SENSITIVITY

The minimum detectable dose of IFN- $\gamma$  was determined by adding two standard deviations to the mean optical density value of the 20 zero standard replicates and calculating the corresponding concentration from the standard curve. The minimum detectable dose using a standard curve generated with Calibrator Diluent I is 13.1 pg/mL and using Calibrator Diluent II is 7.5 pg/mL.

### 5. SPECIFICITY

This sandwich ELISA recognizes both natural and recombinant human IFN- $\gamma$ . This kit exhibits no detectable cross-reactivity with human; SAA, EGF, IL-6, IL-8, IL-16, M-CSF, GM-CSF, TGF- $\beta_1$ , RANTES, FGF, MIP-1 $\alpha$ , CRP, EPO.

## 6. CALIBRATION

This immunoassay is calibrated against British Standard, Code No. 82/587.

## 7. EXPECTED NORMAL VALUES

Biological samples from apparently healthy, normal individuals were collected and the average IFN- $\gamma$  concentration measured. Serum/plasma samples (n=17) are less than 25 pg/mL. Urine samples (n=20) are less than 25 pg/mL

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