

# Quantikine<sup>®</sup>

## Human IFN- $\gamma$ Immunoassay

Catalog Number DIF50

SIF50

PDIF50

**For the quantitative determination of human interferon gamma (IFN- $\gamma$ ) concentrations in cell culture supernates, serum, and plasma.**

*This package insert must be read in its entirety before using this product.*

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

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## INTRODUCTION

Interferon gamma (IFN- $\gamma$ ) is a multifunctional protein first observed as an antiviral activity in cultures of Sindbis virus-infected human leukocytes stimulated by PHA (1). Produced by 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, upregulates class I and II MHC complex antigens and increases the efficiency of macrophage-mediated killing of intracellular parasites (2, 3). 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  $\beta$  (4). Human IFN- $\gamma$  is reported to be active only on human and non-human primate cells (5). The biochemistry and biological activities of the interferons have been extensively reviewed (2 - 9).

Human IFN- $\gamma$  is a 143 amino acid residue, 20 or 25 kDa glycoprotein that demonstrates little sequence homology to IFN- $\alpha$  or - $\beta$  (10 - 13). 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 Asn 97 (13,14). In neither case is glycosylation required for biological activity (15,16). Two allelic variants of IFN- $\gamma$  have been described differing by the presence of an Arg or a Gln at position 137 (10,17). 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 (11). The secreted form of IFN- $\gamma$  has no potential for the formation of disulfide bridges (13). 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 (18,19).

A receptor for IFN- $\gamma$  has been identified and its gene localized to chromosome 6 (20, 21). Apparently the product of a single gene, the receptor is a single chain 90 kDa glycoprotein that shows a high degree of species-specific binding of IFN- $\gamma$  (22 - 25). The cDNA for the receptor encodes a polypeptide with a 17 amino acid residue signal peptide, a 228 residue extracellular domain, a 21 residue transmembrane domain, and a 223 residue intracellular domain. The predicted sequence shows the potential for extensive N- and O-linked glycosylation of the receptor (22). A soluble form of the IFN- $\gamma$  receptor has been found in human urine under normal physiological conditions (26). Evidence indicates that at least one additional receptor component, tentatively designated IFN- $\gamma$  R $\beta$  chain, is required for signal transduction following binding of IFN- $\gamma$  (27 - 30). Binding of IFN- $\gamma$  to its receptor apparently induces dimerization of the receptor and activation of the tyrosine kinases JAK1 and JAK2. The IFN- $\gamma$  receptor and an additional protein, STAT91 (or GAF), are subsequently phosphorylated. Phosphorylated STAT91 dimerizes and, possibly in combination with another protein, enters the cell nucleus where the complex binds to distinct sites within the promoters of IFN- $\gamma$  responsive genes, *e.g.*, the gamma interferon activation site (GAS) and the interferon-stimulated response element (ISRE) (31 - 35). This binding induces the expression of at least 20 distinct proteins, 12 of which are unique to IFN- $\gamma$  stimulation (36, 37).

Functionally, IFN- $\gamma$  produces a variety of effects. Produced by CD8<sup>+</sup>, NK,  $\gamma\delta$ , and TH1 T helper cells, IFN- $\gamma$  has documented antiviral, antiprotozoal and immunomodulatory effects on cell proliferation and apoptosis, as well as the stimulation and repression of a variety of genes (9, 38 - 41). 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- $\gamma$  (42). The immunomodulatory effects of IFN- $\gamma$  are extensive and diverse. In monocyte/macrophages, the activities of IFN- $\gamma$  include: increasing the expression of class I and II MHC antigens; increasing the production of IL-1, platelet-activating factor, H<sub>2</sub>O<sub>2</sub>, and pterin; protection of monocytes against LAK cell-mediated lysis; downregulation of IL-8 mRNA expression that is upregulated by IL-2; and, with lipopolysaccharide, induction of NO production (41 - 44). IFN- $\gamma$  has also been demonstrated to be chemotactic for monocytes but not neutrophils (45). IFN- $\gamma$  selectively enhances both IgG<sub>2a</sub> secretion by LPS-stimulated B cells and IgG<sub>3</sub> secretion in T cell-independent type 2 antigen-mediated B cell activation (46, 47). It has also been reported to induce its own expression (48). Finally, IFN- $\gamma$  has been shown to upregulate ICAM-1, but not E-Selectin or VCAM-1, expression on endothelial cells (49).

The Quantikine Human IFN- $\gamma$  Immunoassay is a 4.5 hour solid phase ELISA designed to measure IFN- $\gamma$  levels in cell culture supernates, serum, and plasma. It contains *E. coli*-expressed recombinant human IFN- $\gamma$  and antibodies raised against the recombinant factor. Results obtained for naturally occurring human IFN- $\gamma$  samples showed linear curves that were parallel to the standard curves obtained using the Quantikine kit standards. These results indicate that this kit can be used to determine relative mass values for natural human IFN- $\gamma$ .

The presence of a naturally occurring form of a receptor for IFN- $\gamma$  has been reported in normal human urine (26). Until the soluble IFN- $\gamma$  receptor has been tested in the Quantikine Human IFN- $\gamma$  Immunoassay, the possibility of interference cannot be excluded.

## **PRINCIPLE OF THE ASSAY**

This assay employs the quantitative sandwich enzyme immunoassay technique. A polyclonal antibody specific for IFN- $\gamma$  has been pre-coated onto a microplate. Standards and samples are pipetted into the wells and any IFN- $\gamma$  present is bound by the immobilized antibody. After washing away any unbound substances, an enzyme-linked polyclonal antibody specific for IFN- $\gamma$  is added to the wells. Following a wash to remove any unbound antibody-enzyme reagent, a substrate solution is added to the wells and color develops in proportion to the amount of IFN- $\gamma$  bound in the initial step. The color development is stopped and the intensity of the color is measured.

## LIMITATIONS OF THE PROCEDURE

- FOR RESEARCH USE ONLY. NOT FOR USE IN DIAGNOSTIC PROCEDURES.
- The kit should not be used beyond the expiration date on the kit label.
- Do not mix or substitute reagents with those from other lots or sources.
- If samples generate values higher than the highest standard, dilute the samples with Calibrator Diluent and repeat the assay. If cell culture supernate samples require large dilution, perform an intermediate dilution in culture media and the final dilution in Calibrator Diluent.
- Any variation in standard diluent, operator, pipetting technique, washing technique, incubation time or temperature, and kit age can cause variation in binding.
- This assay is designed to eliminate interference by soluble receptors, binding proteins, and other factors present in biological samples. Until all factors have been tested in the Quantikine Immunoassay, the possibility of interference cannot be excluded.

## MATERIALS PROVIDED

Description	Part #	Cat. # DIF50	Cat. # SIF50
<b>IFN-<math>\gamma</math> Microplate</b> - 96 well polystyrene microplate (12 strips of 8 wells) coated with a polyclonal antibody against IFN- $\gamma$ .	890582	1 plate	6 plates
<b>IFN-<math>\gamma</math> Conjugate</b> - 21 mL/vial of polyclonal antibody against IFN- $\gamma$ conjugated to horseradish peroxidase with preservatives.	890583	1 vial	6 vials
<b>IFN-<math>\gamma</math> Standard</b> - Recombinant human IFN- $\gamma$ in a buffered protein base with preservatives; lyophilized.	890210	1 vial	6 vials
<b>Assay Diluent RD1-51</b> - 11mL/vial of a buffered protein base with preservatives and blue dye.	895342	1 vial	6 vials
<b>Calibrator Diluent RD6-21</b> - 21 mL/vial of a buffered protein base with preservatives.	895261	1 vial	6 vials
<b>Wash Buffer Concentrate</b> - 21 mL/vial of a 25-fold concentrated solution of buffered surfactant with preservatives.	895003	1 vial	6 vials
<b>Color Reagent A</b> - 12.5 mL/vial of stabilized hydrogen peroxide.	895000	1 vial	6 vials
<b>Color Reagent B</b> - 12.5 mL/vial of stabilized chromogen (tetramethylbenzidine).	895001	1 vial	6 vials
<b>Stop Solution</b> - 6 mL/vial of 2 N sulfuric acid.	895032	1 vial	6 vials
<b>Plate Covers</b> - Adhesive strips.	—	4 strips	24 strips

DIF50 contains sufficient materials to run an ELISA on one 96 well plate.

SIF50 (SixPak) contains sufficient materials to run ELISAs on six 96 well plates.

This kit is also available in a PharmPak (R&D Systems, Catalog # PDIF50). PharmPaks contain sufficient materials to run ELISAs on 50 microplates. Specific vial counts of each component may vary. Please refer to the literature accompanying your order for specific vial counts.

## STORAGE

<b>Unopened Kit</b>	Store at 2 - 8° C. Do not use past kit expiration date.	
<b>Opened/ Reconstituted Reagents</b>	Diluted Wash Buffer	May be stored for up to 1 month at 2 - 8° C.*
	Stop Solution	
	Calibrator Diluent RD6-21	
	Assay Diluent RD1-51	
	Conjugate	
	Unmixed Color Reagent A	
	Unmixed Color Reagent B	
	Standard	Aliquot and store for up to 1 month at $\leq -20^{\circ}\text{C}$ in a manual defrost freezer.* Avoid repeated freeze-thaw cycles.
	Microplate Wells	Return unused wells to the foil pouch containing the desiccant pack, reseal along entire edge of zip-seal. May be stored for up to 1 month at 2 - 8° C.*

\*Provided this is within the expiration date of the kit.

## OTHER SUPPLIES REQUIRED

- Microplate reader capable of measuring absorbance at 450 nm, with the correction wavelength set at 540 nm or 570 nm.
- Pipettes and pipette tips.
- 500 mL graduated cylinder.
- Deionized or distilled water.
- Squirt bottle, manifold dispenser, or automated microplate washer.
- **Polypropylene tubes.**
- Human IFN- $\gamma$  controls (optional; available from R&D Systems).

## PRECAUTION

The Stop Solution provided with this kit is an acid solution. Wear eye, hand, face, and clothing protection when using this material.

## SAMPLE COLLECTION AND STORAGE

**Cell Culture Supernates** - Remove particulates by centrifugation and assay immediately or aliquot and store samples at  $\leq -20^{\circ}\text{C}$ . Avoid repeated freeze-thaw cycles.

**Serum** - Use a serum separator tube (SST) and allow samples to clot for 30 minutes before centrifugation for 10 minutes at  $1000 \times g$ . Remove serum and assay immediately or aliquot and store samples at  $\leq -20^{\circ}\text{C}$ . Avoid repeated freeze-thaw cycles.

**Plasma** - Collect plasma using EDTA as an anticoagulant. Centrifuge at  $1000 \times g$  within 30 minutes of collection. Assay immediately or aliquot and store samples at  $\leq -20^{\circ}\text{C}$ . Avoid repeated freeze-thaw cycles. **Heparin and citrate plasma samples cannot be used in this assay.**

**Note:** Hemolyzed samples are not suitable for the measurement of human IFN- $\gamma$  with this assay. Samples with high levels of rheumatoid factor may interfere with the measurement of human IFN- $\gamma$  in this assay.

## REAGENT PREPARATION

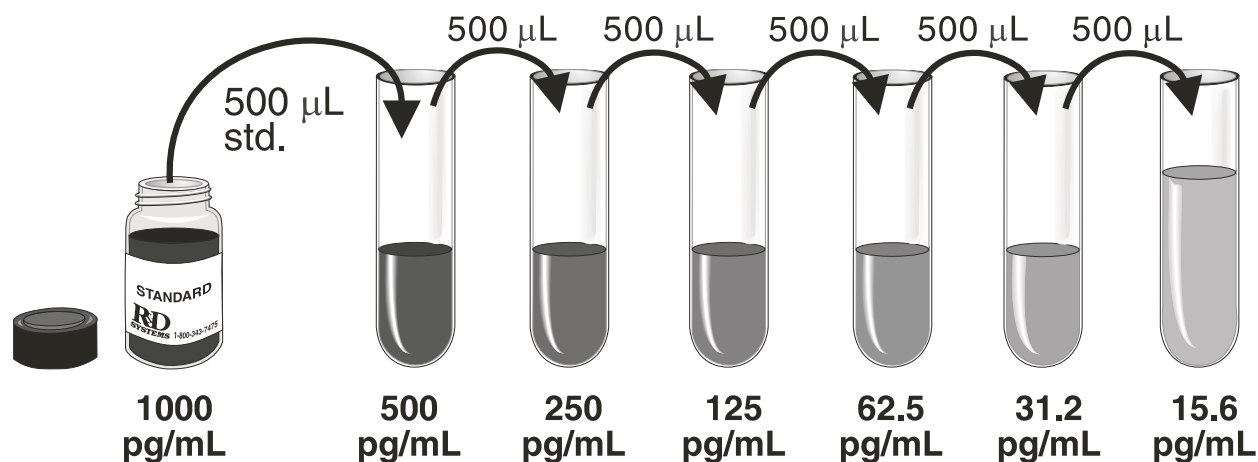
**Bring all reagents to room temperature before use.**

**Wash Buffer** - If crystals have formed in the concentrate, warm to room temperature and mix gently until the crystals have completely dissolved. Dilute 20 mL of Wash Buffer Concentrate into deionized or distilled water to prepare 500 mL of Wash Buffer.

**Substrate Solution** - Color Reagents A and B should be mixed together in equal volumes within 15 minutes of use. Protect from light. 200  $\mu\text{L}$  of the resultant mixture is required per well.

**IFN- $\gamma$  Standard** - Refer to the vial label for reconstitution volume. Reconstitute the IFN- $\gamma$  Standard with Calibrator Diluent RD6-21. This reconstitution produces a stock solution of 1000 pg/mL. Allow the standard to sit for a minimum of 15 minutes with gentle agitation prior to making dilutions.

**Use polypropylene tubes.** Pipette 500  $\mu\text{L}$  of Calibrator Diluent RD6-21 into each tube. Use the stock solution to produce a dilution series (below). Mix each tube thoroughly before the next transfer. The undiluted standard serves as the high standard (1000 pg/mL). Calibrator Diluent RD6-21 serves as the zero standard (0 pg/mL).



## ASSAY PROCEDURE

**Bring all reagents and samples to room temperature before use. It is recommended that all samples, standards, and control be assayed in duplicate.**

1. Prepare all reagents and working standards as directed in the previous sections.
2. Remove excess microplate strips from the plate frame, return them to the foil pouch containing the desiccant pack, reseal.
3. Add 100  $\mu\text{L}$  of Assay Diluent RD1-51 to each well.
4. Add 100  $\mu\text{L}$  of Standard, sample, or control per well. **Ensure reagent addition is uninterrupted and completed within 15 minutes.** Cover with the adhesive strip provided. Incubate for 2 hours at room temperature. A plate layout is provided to record standards and samples assayed.
5. Aspirate each well and wash, repeating the process three times for a total of four washes. Wash by filling each well with Wash Buffer (400  $\mu\text{L}$ ) using a squirt bottle, manifold dispenser, or autowasher. Complete removal of liquid at each step is essential to good performance. After the last wash, remove any remaining Wash Buffer by aspirating or decanting. Invert the plate and blot it against clean paper towels.
6. Add 200  $\mu\text{L}$  of IFN- $\gamma$  Conjugate to each well. Cover with a new adhesive strip. Incubate for 2 hours at room temperature.
7. Repeat the aspiration/wash as in step 5.
8. Add 200  $\mu\text{L}$  of Substrate Solution to each well. Incubate for 30 minutes at room temperature. **Protect from light.**
9. Add 50  $\mu\text{L}$  of Stop Solution to each well. The color in the well should change from blue to yellow. If the color in the well is green or if the color change does not appear uniform, gently tap the plate to ensure thorough mixing.
10. Determine the optical density of each well within 30 minutes, using a microplate reader set to 450 nm. If wavelength correction is available, set to 540 nm or 570 nm. If wavelength correction is not available, subtract readings at 540 nm or 570 nm from the readings at 450 nm. This subtraction will correct for optical imperfections in the plate. Readings made directly at 450 nm without correction may be higher and less accurate.

## ASSAY PROCEDURE SUMMARY

1. Prepare all reagents and standards as directed.



2. Add 100  $\mu\text{L}$  Assay Diluent RD1-51 to each well.



3. Add 100  $\mu\text{L}$  Standard, sample, or control to each well **within 15 minutes**. Incubate 2 hrs. RT



4. Aspirate and wash 4 times.



5. Add 200  $\mu\text{L}$  Conjugate to each well.  
Incubate 2 hrs. RT



6. Aspirate and wash 4 times.



7. Add 200  $\mu\text{L}$  Substrate Solution to each well.  
Incubate 30 min. RT. **Protect from light.**



8. Add 50  $\mu\text{L}$  Stop Solution to each well.  
Read at 450 nm within 30 minutes.  
 $\lambda$  correction 540 or 570 nm

## CALCULATION OF RESULTS

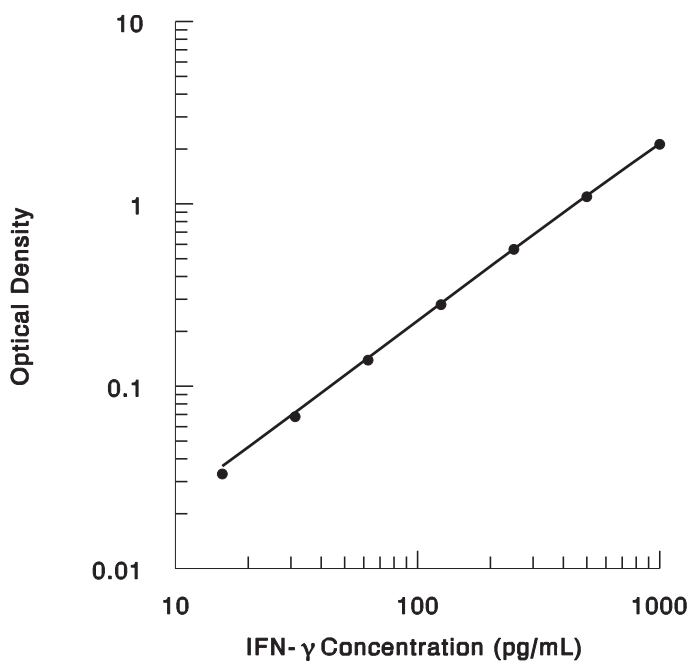
Average the duplicate readings for each standard, control, and sample and subtract the average zero standard optical density.

Create a standard curve by reducing the data using computer software capable of generating a four parameter logistic (4-PL) curve-fit. As an alternative, construct a standard curve by plotting the mean absorbance for each standard on the y-axis against the concentration on the x-axis and draw a best fit curve through the points on the graph. The data may be linearized by plotting the log of the IFN- $\gamma$  concentrations versus the log of the O.D. and the best fit line can be determined by regression analysis. This procedure will produce an adequate but less precise fit of the data.

If samples have been diluted, the concentration read from the standard curve must be multiplied by the dilution factor.

## TYPICAL DATA

This standard curve is provided for demonstration only. A standard curve should be generated for each set of samples assayed.



(pg/mL)	O.D.	Average	Corrected
0	0.094 0.093 0.125	0.094	—
15.6	0.129 0.163	0.127	0.033
31.2	0.162 0.231	0.162	0.068
62.5	0.235 0.373	0.233	0.139
125	0.376 0.644	0.374	0.280
250	0.670 1.190	0.657	0.563
500	1.187 2.196	1.188	1.094
1000	2.237	2.216	2.122

## TECHNICAL HINTS

- When mixing or reconstituting protein solutions, always avoid foaming.
- To avoid cross-contamination, change pipette tips between additions of each standard level, between sample additions, and between reagent additions. Also, use separate reservoirs for each reagent.
- When using an automated plate washer, adding a 30 second soak period following the addition of wash buffer, and/or rotating the plate 180 degrees between wash steps may improve assay precision.
- To ensure accurate results, proper adhesion of plate sealers during incubation steps is necessary.
- Substrate Solution should remain colorless until added to the plate. Keep Substrate Solution protected from light. Substrate Solution should change from colorless to gradations of blue.
- Stop Solution should be added to the plate in the same order as the Substrate Solution. The color developed in the wells will turn from blue to yellow upon addition of the Stop Solution. Wells that are green in color indicate that the Stop Solution has not mixed thoroughly with the Substrate Solution.

## PRECISION

### Intra-assay Precision (Precision within an assay)

Three samples of known concentration were tested twenty times on one plate to assess intra-assay precision.

### Inter-assay Precision (Precision between assays)

Three samples of known concentration were tested in forty separate assays to assess inter-assay precision.

Sample	Intra-assay Precision			Inter-assay Precision		
	1	2	3	1	2	3
n	20	20	20	40	40	40
Mean (pg/mL)	79.2	214	466	78.7	206	457
Standard deviation	3.7	5.5	13.0	6.1	13.1	17.0
CV (%)	4.7	2.6	2.8	7.8	6.4	3.7

## RECOVERY

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

Sample Type	Average % Recovery	Range
Cell culture media	102	93 - 109%
Serum	102	91 - 118%
EDTA plasma	98	88 - 111%

## LINEARITY

To assess the linearity of the assay, five samples were spiked with high concentrations of IFN- $\gamma$  in various matrices and diluted with Calibrator Diluent RD6-21 to produce samples with values within the dynamic range of the assay.

		Cell culture media	Serum	EDTA plasma
1:2	Average % of Expected	98	100	104
	Range (%)	95-103	98-101	101-109
1:4	Average % of Expected	100	99	107
	Range (%)	93-106	97-103	101-115
1:8	Average % of Expected	98	96	101
	Range (%)	88-109	92-99	94-107
1:16	Average % of Expected	93	89	94
	Range (%)	75-109	84-98	86-99

## SENSITIVITY

The minimum detectable dose of IFN- $\gamma$  is typically less than 8.0 pg/mL.

The minimum detectable dose was determined by adding two standard deviations to the mean optical density value of twenty zero standard replicates and calculating the corresponding concentration.

## CALIBRATION

This immunoassay is calibrated against a highly purified *E. coli*-expressed recombinant human IFN- $\gamma$  produced at R&D Systems. The NIBSC/WHO 1<sup>st</sup> British Standard for human leukocyte IFN- $\gamma$  82/587 was evaluated in this kit in July 2008. To convert sample values obtained with the Quantikine Human IFN- $\gamma$  kit to relative equivalent NIBSC units, use the equation below:

$$\text{NIBSC/WHO (82/587) equivalent value (U/mL)} = 0.017 \times \text{Quantikine IFN-}\gamma \text{ value (pg/mL)}$$

## SAMPLE VALUES

**Serum/Plasma** - Serum and plasma samples drawn from thirty apparently healthy volunteers were evaluated for the presence of IFN- $\gamma$  in this assay. All samples measured less than the lowest IFN- $\gamma$  standard, 15.6 pg/mL. No medical histories were available for the donors used in this study.

**Cell Culture Supernates** - Human peripheral blood mononuclear cells ( $1 \times 10^6$  cells/mL) were cultured in RPMI supplemented with 10% fetal calf serum, 50  $\mu$ M  $\beta$ -mercaptoethanol, 2 mM L-glutamine, 100 U/mL penicillin, and 100  $\mu$ g/mL streptomycin sulfate. Cells were cultured unstimulated or stimulated with 10  $\mu$ g/mL of PHA. Aliquots of the culture supernate were removed on days 1 and 5 and assayed for levels of natural IFN- $\gamma$ .

Condition	Day 1 (pg/mL)	Day 5 (pg/mL)
Unstimulated	453	2651
Stimulated	8360	7851

## SPECIFICITY

This assay recognizes both natural and recombinant human IFN- $\gamma$ . The factors listed below were prepared at 50 ng/mL in Calibrator Diluent RD6-21 and assayed for cross-reactivity. Preparations of the following factors at 50 ng/mL in a mid-range rhIFN- $\gamma$  control were assayed for interference. No significant cross-reactivity or interference was observed.

### Recombinant human:

Amphiregulin	IL-6 sR
Angiogenin	IL-7
CNTF	IL-8
$\beta$ -ECGF	IL-9
EGF	IL-10
Epo	IL-11
FGF acidic	IL-12
FGF basic	IL-13
FGF-4	KGF (FGF-7)
FGF-5	LAP (TGF- $\beta$ 1)
FGF-6	LIF
G-CSF	M-CSF
GM-CSF	MCP-1
sgp130	MIP-1 $\alpha$
GRO $\alpha$	MIP-1 $\beta$
GRO $\beta$	$\beta$ -NGF
GRO $\gamma$	Oncostatin M
HB-EGF	PD-ECGF
HGF	PDGF-AA
IGF-I	PDGF-AB
IGF-II	PDGF-BB
IL-1 $\alpha$	PTN
IL-1 $\beta$	RANTES
IL-1ra	SCF
IL-1 sR $\alpha$	SLPI
IL-1 sR $\beta$	TGF- $\alpha$
IL-1 sRII	TGF- $\beta$ 1
IL-2	TGF- $\beta$ 2
IL-2 sR $\alpha$	TGF- $\beta$ 3
IL-3	TGF- $\beta$ 5
IL-3 sR $\alpha$	TGF- $\beta$ sRII
IL-4	TNF- $\alpha$
IL-4 sR	TNF- $\beta$
IL-5	sTNF RI
IL-5 sR $\alpha$	sTNF RII
IL-6	VEGF

### Recombinant mouse:

GM-CSF  
IFN- $\gamma$   
IL-1 $\alpha$   
IL-1 $\beta$   
IL-3  
IL-4  
IL-5  
IL-6  
IL-7  
IL-9  
IL-10  
IL-13  
LIF  
MIP-1 $\alpha$   
MIP-1 $\beta$   
SCF  
TNF- $\alpha$

### Recombinant rat:

IFN- $\gamma$

### Recombinant amphibian:

TGF- $\beta$ 5

### Recombinant chicken:

TGF- $\beta$ 3

### Other proteins:

mouse EGF  
bovine FGF acidic  
bovine FGF basic  
human PDGF  
porcine PDGF  
human TGF- $\beta$ 1  
porcine TGF- $\beta$ 1

Recombinant rhesus macaque IFN- $\gamma$  was found to cross-react approximately 20%.

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# PLATE LAYOUT

Use this plate layout to record standards and samples assayed.

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