

Quantikine[®] ELISA

Human IFN- γ Immunoassay

Catalog Number DIF50C

SIF50C

PDIF50C

For the quantitative determination of human interferon gamma (IFN- γ) 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- γ) is an important immunomodulatory cytokine, affecting both the innate and adaptive immune systems. It was discovered in 1965 as a soluble anti-viral factor and has since been shown to promote host defense against a wide variety of pathogens (1, 2). Additionally, it has been shown to promote autophagy and apoptosis, and to have anti-proliferative, anti-angiogenic, and anti-tumorigenic properties (1, 3, 4). IFN- γ is primarily secreted by natural killer (NK) cells (5-7), activated CD8⁺ T cells (8), Th1 CD4⁺ T cells (9), NKT cells (10, 11), and macrophages (12-16), but it has also been shown to be produced by a number of other cell types including dendritic cells (17), $\gamma\delta$ T cells (18), group 1 ILCs (19), keratinocytes (20), neutrophils (21), mast cells (22), and neurons (23).

The biologically active form of IFN- γ is a non-covalently linked homodimer (24), which binds with high affinity to IFN- γ R1/CD119 and subsequently recruits IFN- γ R2 to form the functional heterotetrameric receptor complex. Formation of this complex leads to phosphorylation and activation of the Janus kinases, Jak1 and Jak2, which in turn phosphorylate and activate STAT1. STAT1 homodimerizes and translocates to the nucleus where it binds to IFN- γ -activated sequence (GAS) elements in the promoters of target genes to regulate their transcription. Many of the IFN- γ /STAT1 target genes are transcription factors that then drive the expression of secondary response genes. Additionally, IFN- γ signaling has been shown to activate MAPK, PI 3-K/Akt, and the NF- κ B signaling pathways, leading to the expression of multiple other genes. IFN- γ signaling plays a key role in host defense by promoting macrophage activation, upregulating the expression of antigen processing and presentation molecules, driving the development and activation of Th1 cells, enhancing natural killer cell activity, regulating B cell functions, and inducing the production of chemokines that promote effector cell trafficking to sites of inflammation.

Due to its immunoregulatory activities, IFN- γ has been used as a therapeutic agent for treating a range of bacterial, fungal, helminth, protozoan, and viral infections, immunodeficiency syndromes, multi-drug resistant tuberculosis (MDR-TB), and sepsis (1, 25-34). Additionally, it has been used as an anti-tumor agent to improve patient survival in a number of different types of cancer due to its pro-apoptotic and anti-angiogenic effects (3, 35). In contrast, IFN- γ has been suggested to be involved in the progression of cardiac diseases as elevated levels of this cytokine have been detected in the serum of patients with chronic heart failure, as well as in atherosclerotic lesions and in myocardial tissues of patients with Chagas' cardiomyopathy (1, 36, 37). Similarly, high levels of IFN- γ have been found in the serum and/or cerebrospinal fluid of patients with neurodegenerative diseases such as Amyotrophic lateral sclerosis and Parkinson's disease (38, 39), suggesting that IFN- γ may also be involved in neurodegenerative disease progression and serve as a clinical biomarker. Additionally, there is recent evidence suggesting that IFN- γ may also have context-dependent proliferative and pro-tumorigenic effects (3).

The Quantikine[®] Human IFN- γ Immunoassay is a 4.5 hour solid phase ELISA designed to measure human IFN- γ levels in cell culture supernates, serum, and plasma. It contains HEK293-expressed recombinant human IFN- γ and antibodies raised against the recombinant factor. Results obtained for naturally occurring human IFN- γ 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- γ .

PRINCIPLE OF THE ASSAY

This assay employs the quantitative sandwich enzyme immunoassay technique. A monoclonal antibody specific for human IFN- γ has been pre-coated onto a microplate. Standards and samples are pipetted into the wells and any IFN- γ present is bound by the immobilized antibody. After washing away any unbound substances, an enzyme-linked monoclonal antibody specific for human IFN- γ 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- γ 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.
- Any variation in diluent, operator, pipetting technique, washing technique, incubation time or temperature, and kit age can cause variation in binding.
- Variations in sample collection, processing, and storage may cause sample value differences.
- This assay is designed to eliminate interference by other factors present in biological samples. Until all factors have been tested in the Quantikine® Immunoassay, the possibility of interference cannot be excluded.

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.
- To ensure accurate results, proper adhesion of plate sealers during incubation steps is necessary.
- 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.
- 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.

MATERIALS PROVIDED & STORAGE CONDITIONS

Store the unopened kit at 2-8 °C. Do not use past kit expiration date.

PART	PART #	CATALOG # DIF50C	CATALOG # SIF50C	DESCRIPTION	STORAGE OF OPENED/ RECONSTITUTED MATERIAL
Human IFN- γ Microplate	899182	1 plate	6 plates	96 well polystyrene microplate (12 strips of 8 wells) coated with a monoclonal antibody specific for human IFN- γ .	Return unused wells to the foil pouch containing the desiccant pack. Reseal along entire edge of the zip-seal. May be stored for up to 1 month at 2-8 °C.*
Human IFN- γ Standard	899184	2 vials	12 vials	Recombinant human IFN- γ in a buffered protein base with preservatives; lyophilized. <i>Refer to the vial label for reconstitution volume.</i>	Use a new standard for each assay. Discard after use.
Human IFN- γ Conjugate	899183	1 vial	6 vials	21 mL/vial of a monoclonal antibody specific for human IFN- γ conjugated to horseradish peroxidase with preservatives.	May be stored for up to 1 month at 2-8 °C.*
Assay Diluent RD1-63	895352	1 vial	6 vials	12 mL/vial of a buffered protein base with preservatives.	
Calibrator Diluent RD5P	895151	1 vial	6 vials	21 mL/vial of a buffered protein base with preservatives. <i>Use diluted 1:5 in this assay.</i>	
Wash Buffer Concentrate	895003	1 vial	6 vials	21 mL/vial of a 25-fold concentrated solution of buffered surfactant with preservatives. <i>May turn yellow over time.</i>	
Color Reagent A	895000	1 vial	6 vials	12 mL/vial of stabilized hydrogen peroxide.	
Color Reagent B	895001	1 vial	6 vials	12 mL/vial of stabilized chromogen (tetramethylbenzidine).	
Stop Solution	895032	1 vial	6 vials	6 mL/vial of 2 N sulfuric acid.	
Plate Sealers	N/A	4 strips	24 strips	Adhesive strips.	

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

DIF50C contains sufficient materials to run an ELISA on one 96 well plate.

SIF50C (SixPak) contains sufficient materials to run ELISAs on six 96 well plates.

This kit is also available in a PharmPak (R&D Systems®, Catalog # PDIF50C). Refer to the PharmPak Contents section for specific vial counts.

PHARMPAK CONTENTS

Each PharmPak contains reagents sufficient for the assay of 50 microplates (96 wells/plate). The package inserts supplied are the same as those supplied in the single kit packs and because of this, a few minor differences related to the number of reagents and their container sizes should be noted.

- Sufficient material is supplied to perform at least 50 standard curves; reuse of each vial may be required. The number of vials, and the number of standard curves obtained per vial will vary with the analyte.
- Wash Buffer 25X Concentrate is bulk packed in 125 mL bottles containing 100 mL.
Note: *Additional wash buffer is available for purchase (R&D Systems®, Catalog # WA126).*

The reagents provided in this PharmPak are detailed below.

PART	PART #	QUANTITY
Human IFN- γ Microplate	899182	50 plates
Human IFN- γ Standard	899184	25 vials
Human IFN- γ Conjugate	899183	50 vials
Assay Diluent RD1-63	895352	50 vials
Calibrator Diluent RD5P	895151	25 vials
Wash Buffer Concentrate	895126	9 bottles
Color Reagent A	895000	50 vials
Color Reagent B	895001	50 vials
Stop Solution	895032	50 vials
Plate Sealers	N/A	100 sheets
Package Insert	753349	2 booklets

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
- 50 mL and 500 mL graduated cylinders
- Deionized or distilled water
- Squirt bottle, manifold dispenser, or automated microplate washer
- Test tubes for dilution of standards
- Human IFN- γ Controls (optional; R&D Systems[®], Catalog # QC281)

PRECAUTIONS

The Stop Solution provided with this kit is an acid solution.

Some components in this kit contain a preservative which may cause an allergic skin reaction. Avoid breathing mist.

Color Reagent B may cause skin, eye, and respiratory irritation. Avoid breathing fumes.

Wear protective gloves, clothing, eye, and face protection. Wash hands thoroughly after handling. Refer to the SDS on our website prior to use.

SAMPLE COLLECTION & STORAGE

The sample collection and storage conditions listed below are intended as general guidelines. Sample stability has not been evaluated.

Cell Culture Supernates - Remove particulates by centrifugation and assay immediately or aliquot and store samples at ≤ -20 °C. Avoid repeated freeze-thaw cycles.

Serum - Use a serum separator tube (SST) and allow samples to clot for 30 minutes at room temperature before centrifugation for 15 minutes at 1000 x g. Remove serum and assay immediately or aliquot and store samples at ≤ -20 °C. Avoid repeated freeze-thaw cycles.

Plasma - Collect plasma using EDTA or heparin as an anticoagulant. Centrifuge for 15 minutes at 1000 x g within 30 minutes of collection. Assay immediately or aliquot and store samples at ≤ -20 °C. Avoid repeated freeze-thaw cycles.

Note: *Citrate plasma has not been validated for this assay.*

REAGENT PREPARATION

Bring all reagents to room temperature before use.

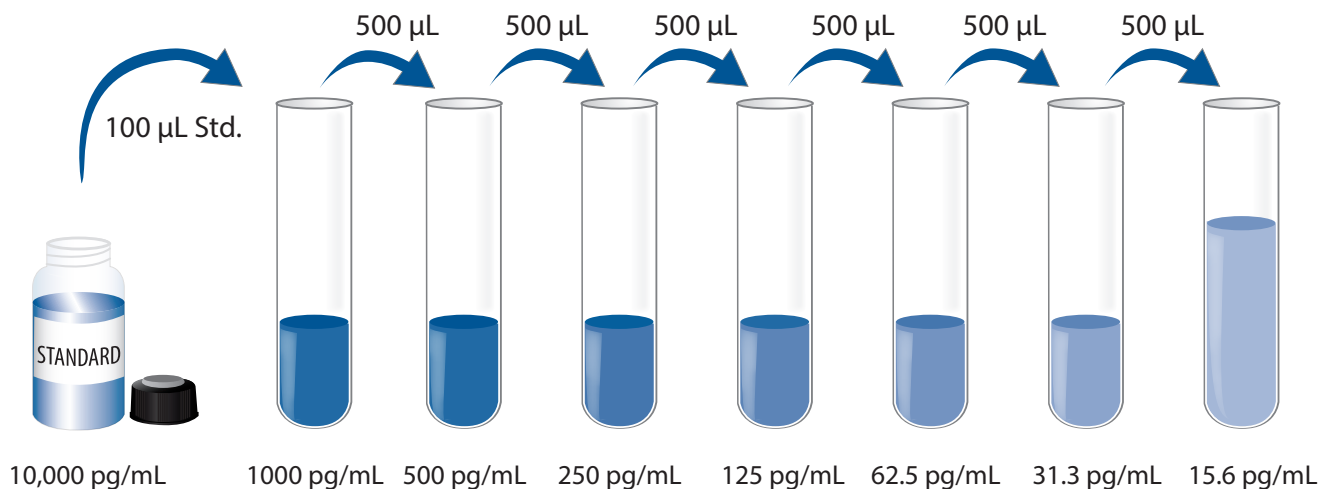
Wash Buffer - If crystals have formed in the concentrate, warm to room temperature and mix gently until the crystals have completely dissolved. Add 20 mL of Wash Buffer Concentrate to 480 mL of deionized or distilled water to prepare 500 mL of Wash Buffer.

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

Calibrator Diluent RD5P (diluted 1:5) - Add 5 mL of Calibrator Diluent RD5P to 20 mL of deionized or distilled water to prepare 25 mL of Calibrator Diluent RD5P (diluted 1:5).

Human IFN- γ Standard - Refer to the vial label for reconstitution volume. Reconstitute the Human IFN- γ Standard with deionized or distilled water. This reconstitution produces a stock solution of 10,000 pg/mL. Allow the standard to sit for a minimum of 15 minutes with gentle agitation prior to making dilutions.

Pipette 900 μ L of Calibrator Diluent RD5P (diluted 1:5) into the 1000 pg/mL tube. Pipette 500 μ L into the remaining tubes. Use the stock solution to produce a dilution series (below). Mix each tube thoroughly before the next transfer. The 1000 pg/mL standard serves as the high standard. Calibrator Diluent RD5P (diluted 1:5) 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 standards, controls, and samples 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, and reseal.
3. Add 100 μL of Assay Diluent RD1-63 to each well.
4. Add 100 μL of standard, control, or sample per well. Cover with the adhesive strip provided. Incubate for 2 hours at room temperature. **Ensure reagent addition is uninterrupted and completed within 15 minutes.** 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 μL) 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 μL of Human IFN- γ 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 μL of Substrate Solution to each well. Incubate for 30 minutes at room temperature. **Protect from light.**
9. Add 50 μL 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.

CALCULATION OF RESULTS

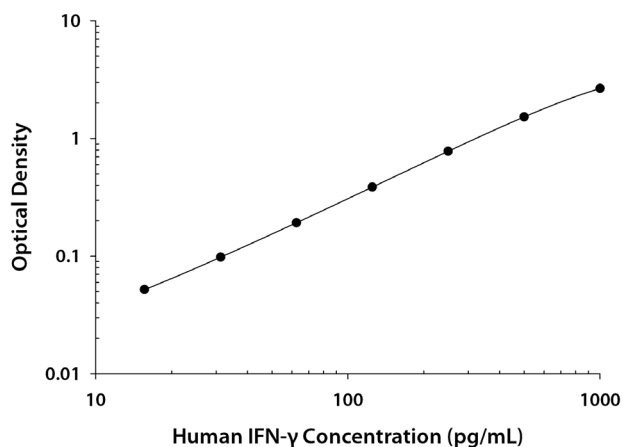
Average the duplicate readings for each standard, control, and sample and subtract the average zero standard optical density (O.D.).

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 human IFN- γ 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	0.009 0.010	0.010	—
15.6	0.050 0.053	0.052	0.042
31.3	0.096 0.099	0.098	0.088
62.5	0.187 0.197	0.192	0.182
125	0.381 0.393	0.387	0.377
250	0.769 0.788	0.779	0.769
500	1.514 1.538	1.526	1.516
1000	2.638 2.693	2.666	2.656

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 twenty separate assays to assess inter-assay precision. Assays were performed by at least three technicians using two lots of components.

Sample	Intra-Assay Precision			Inter-Assay Precision		
	1	2	3	1	2	3
n	20	20	20	20	20	20
Mean (pg/mL)	153	366	720	154	366	723
Standard deviation	3.47	9.16	19.4	9.90	20.6	54.0
CV (%)	2.3	2.5	2.7	6.4	5.6	7.5

RECOVERY

The recovery of human IFN- γ spiked to levels throughout the range of the assay in various matrices was evaluated.

Sample Type	Average % Recovery	Range
Cell culture media (n=4)	91	84-106%
Serum (n=4)	95	90-109%
EDTA plasma (n=4)	95	85-105%
Heparin plasma (n=4)	96	87-101%

SENSITIVITY

Twenty-nine assays were evaluated and the minimum detectable dose (MDD) of human IFN- γ ranges from 0.149-5.69 pg/mL. The mean MDD was 1.28 pg/mL.

The MDD was determined by adding two standard deviations to the mean O.D. value of twenty zero standard replicates and calculating the corresponding concentration.

LINEARITY

To assess the linearity of the assay, samples containing and/or spiked with high concentrations of human IFN- γ in various matrices were diluted with calibrator diluent to produce samples with values within the dynamic range of the assay.

		Cell culture supernates* (n=4)	Serum (n=4)	EDTA plasma (n=4)	Heparin plasma (n=4)
1:2	Average % of Expected	100	107	105	106
	Range (%)	99-103	101-111	102-107	104-109
1:4	Average % of Expected	102	111	109	109
	Range (%)	102-103	105-117	101-114	103-114
1:8	Average % of Expected	105	116	111	111
	Range (%)	104-106	112-120	103-119	106-119
1:16	Average % of Expected	105	115	109	110
	Range (%)	103-107	112-119	101-115	108-112

*Samples were diluted prior to assay.

CALIBRATION

This immunoassay is calibrated against a highly purified HEK293-expressed recombinant human IFN- γ produced at R&D Systems®.

The NIBSC/WHO Human IFN- γ International Standard 82/587 (lectin-stimulated human leukocyte derived) was evaluated in this kit. The dose response curve in this International Standard parallels the Quantikine® standard curve.

To convert sample values obtained with the Quantikine® Human IFN- γ kit to approximate NIBSC/WHO 82/587 International units, use the equation below.

NIBSC/Non-WHO (82/587) approximate value (IU/mL) = 0.0109 x Quantikine® Human IFN- γ value (pg/mL)

Based on data generated from February 2020.

SAMPLE VALUES

Serum/Plasma - Thirty samples from apparently healthy volunteers were evaluated for the presence of human IFN- γ in this assay. No medical histories were available for the donors used in this study. All samples measured less than the lowest human IFN- γ standard, 15.6 pg/mL.

Cell Culture Supernates:

Peripheral blood mononuclear cells from a single donor (PBMCs; seeded at 1×10^6 /mL) were cultured in RPMI 1640 supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 100 U/mL penicillin, and 100 μ g/mL streptomycin sulfate. Cells were left untreated or treated with 10 μ g/mL of PHA for 5 days. Aliquots of the cell culture supernates were removed and assayed for levels of human IFN- γ .

Condition	(pg/mL)
Unstimulated	ND
Stimulated with PHA	78,480

ND=Non-Detectable

CD4⁺ T cells were isolated from human PBMCs (from a single donor) using the MagCelect Human CD4⁺ T cell Isolation Kit (R&D Systems® Catalog# MAGH102). CD4⁺ T cells were then seeded at 5×10^5 /mL and cultured in ExCellerate Human T Cell Expansion Media, Xeno-Free (R&D Systems® Catalog# CCM030). T cells were left unstimulated in culture media, or cultured with 10 ng/mL GMP recombinant human (rh) IL-7 (R&D Systems® Catalog# 207-GMP) and 10 ng/mL GMP rhIL-15 (R&D Systems® Catalog# 247-GMP) and stimulated using immobilized Human CD3 epsilon Antibody (R&D Systems® Catalog# MAB100, coated at 1 μ g/mL) with 5 μ g/mL soluble Human CD28 Antibody (R&D Systems® Catalog# MAB342) or 25 μ L Cloudz CD3/CD28 (Cloudz™ T Cell Activation Kit CD3/CD28, R&D Systems®) per mL of cultured media for 5 days. Aliquots of the cell culture supernates were removed and assayed for levels of human IFN- γ .

Condition	(pg/mL)
Untreated	ND
Stimulated with CD3/CD28 antibodies	49,500
Stimulated with Cloudz T Cell Activation Kit	186,800

ND=Non-Detectable

Sample value may vary from an individual donor.

SPECIFICITY

This assay recognizes natural and recombinant human IFN- γ .

The factors listed below were prepared at 50 ng/mL in calibrator diluent and assayed for cross-reactivity. Preparations of the following factors at 50 ng/mL in a mid-range recombinant human IFN- γ control were assayed for interference. No significant cross-reactivity or interference was observed.

Recombinant human:

IFN- α 1a	IFN- α 14
IFN- α 1b	IFN- α 16
IFN- α 2	IFN- α 17
IFN- α 2a	IFN- α 21
IFN- α 4a	IFN α 2a + IFN α 1b
IFN- α 4b	complex
IFN- α 5	IFN- β
IFN- α 6	IFN- γ R1
IFN- α 7	IFN- γ R2
IFN- α 8	IL-28A
IFN- α 10	IL-28B
	IL-29

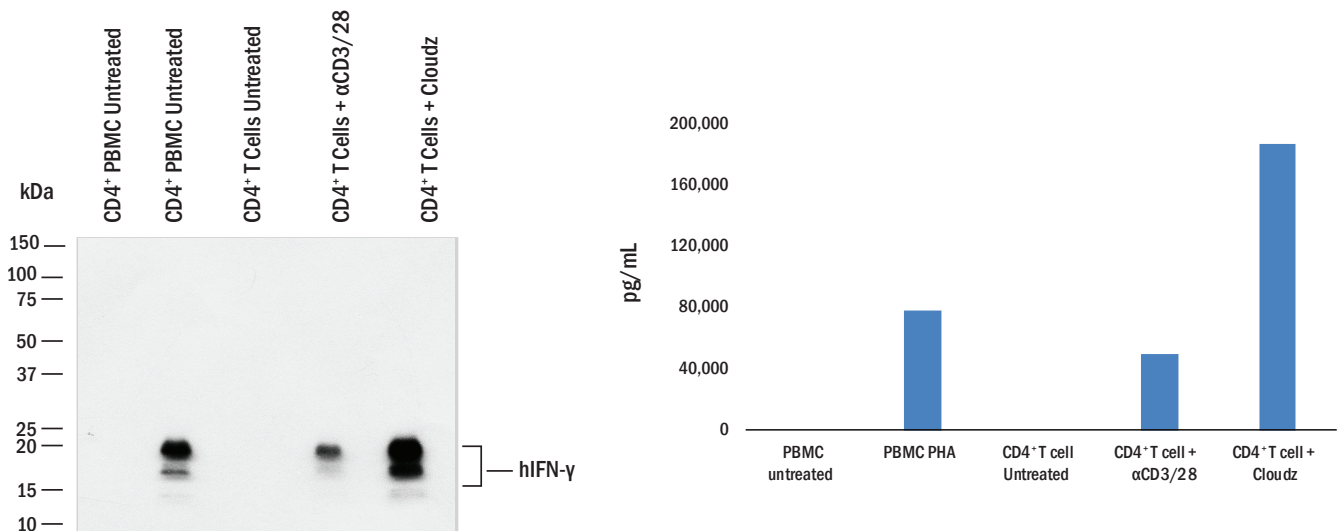
Recombinant mouse:

IFN- γ
IFN- γ R1

Other recombinants:

bovine IFN- γ
canine IFN- γ
cotton rat IFN- γ
equine IFN- γ
feline IFN- γ
porcine IFN- γ
rat IFN- γ

Recombinant rhesus macaque IFN- γ interferes at concentrations > 5 ng/mL and cross-reacts approximately 1.6% in this assay.



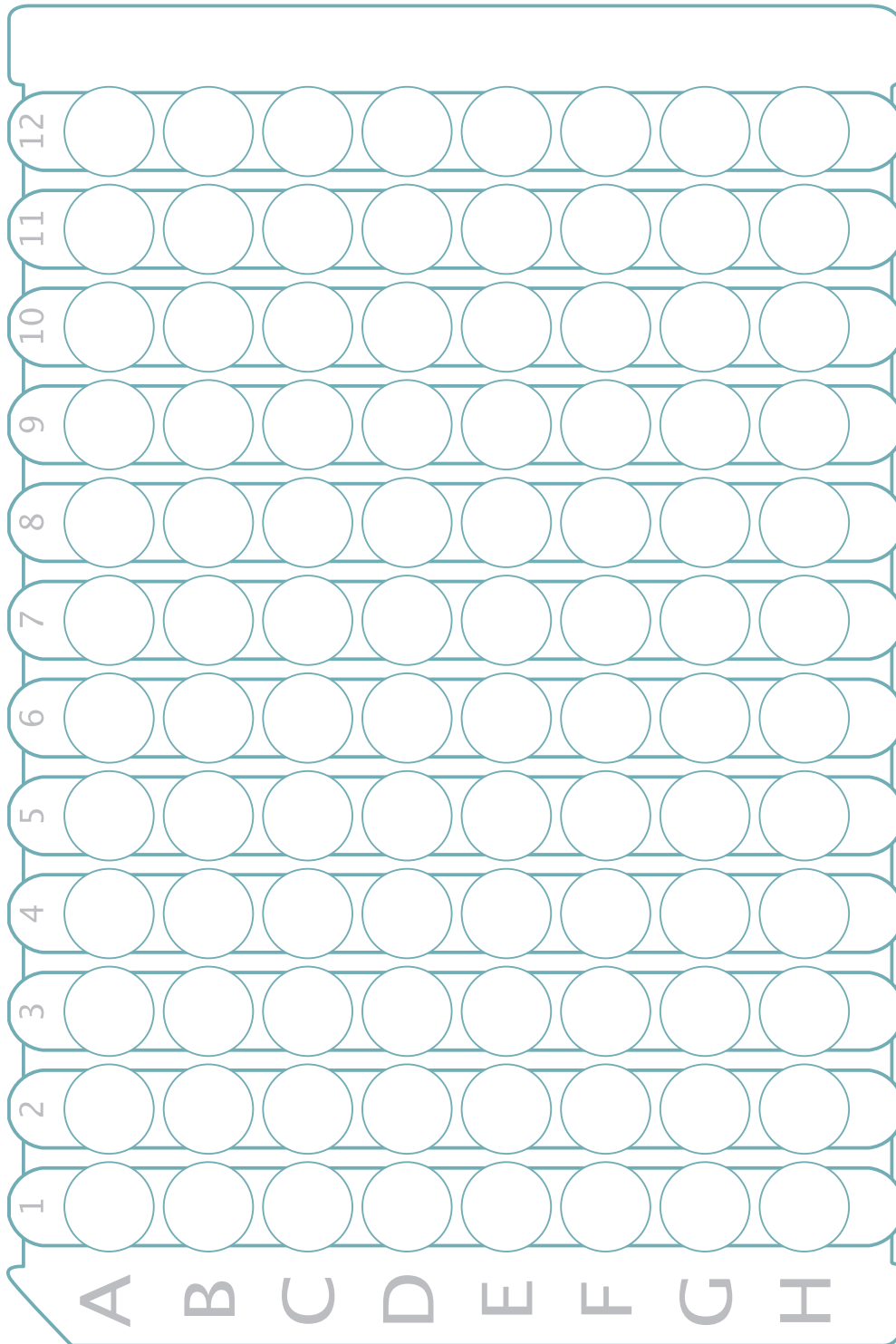
Conditioned media samples were analyzed by Western Blot and Quantikine[®] ELISA. PBMCs were separated from whole blood by a density gradient centrifugation method using Ficoll-Paque Plus (GE Healthcare Catalog # 17-1440-02) and were left untreated or treated with 10 μ g/mL PHA for 5 days. CD4⁺ T cells were further isolated from PBMCs using the MagCelect Human CD4⁺ T cell Isolation Kit (R&D Systems[®], Catalog # MAGH102). T cells were left untreated or treated with 10 ng/mL GMP rhIL-7 (R&D Systems[®], Catalog # 207-GMP), 10 ng/mL GMP rhIL-15 (R&D Systems[®], Catalog # 247-GMP) and stimulated via their T cell receptor (TCR) and co-stimulatory receptor for 5 days. TCR stimulation was mediated using either immobilized Human CD3 ϵ Antibody (R&D Systems[®], Catalog # MAB100) and soluble Human CD28 Antibody (R&D Systems[®], Catalog # MAB342) or Cloudz CD3/28 (Cloudz[™] T Cell Activation Kit – CD3/CD28, R&D Systems[®]). For Western Blot, samples were resolved under reducing SDS-PAGE conditions, transferred to PVDF membrane, and immunoblotted with goat anti-hIFN- γ (R&D Systems[®], Catalog # AF-285-NA). The Western Blot shows a direct correlation with ELISA values for these samples.

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PLATE LAYOUT

Use this plate layout to record standards and samples assayed.



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