BD OptEIA™

Mouse IFN-γ ELISA Kit II

Instruction Manual

Catalog No. 558258

BD

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Introduction

Interferon-γ (IFN-γ) is a potent multifunctional cytokine which is secreted by activated NK cells and $CD4^+TCR\alpha\beta^+$, $CD8^+TCR\alpha\beta^+$, and $TCR\gamma\delta^+$ T cells and exerts its biological effects through specific binding to a single class of high affinity receptors.[1,2] In addition to its antiviral effects, IFN-γ can upregulate a number of lymphoid cell functions including the antimicrobial and antitumor responses of macrophages, NK cells, and neutrophils. IFN-γ exerts strong regulatory influences on the proliferation, differentiation, and effector responses of B cell and T cell subsets. These influences can involve IFN-γ 's capacity to boost MHC class I and II expression by antigen-presenting cells as well as direct effects on B cells and T cells themselves. The BD Biosciences Pharmingen recombinant mouse IFN-γ presents as a 30 kD homodimeric protein, as shown by SDS-PAGE analysis.

Intended Use

The BD OptEIA™ Mouse IFN-γ ELISA Kit II is for the quantitative determination of mouse IFN-γ in serum, plasma, and cell culture supernatant.

Principle of the Test

The BD OptEIA test is a solid phase sandwich ELISA (Enzyme-Linked Immunosorbent Assay). It utilizes a monoclonal antibody specific for mouse IFN-γ coated on a 96-well plate. Standards and samples are added to the wells, and any IFN-γ present binds to the immobilized antibody. The wells are washed and a mixture of biotinylated anti-mouse IFN-γ antibody and streptavidin-horseradish peroxidase is added, producing an antibody-antigen-antibody "sandwich". The wells are again washed and TMB substrate solution is added, which produces a blue color at levels in direct proportion to the amount of IFN-γ present in the initial sample. The Stop Solution changes the color from blue to yellow, and the wells are read at 450 nm.

Reagents Provided

Antibody Coated Wells:	2 plates of 96 breakable wells (12 strips × 8 wells) coated with anti-mouse IFN-γ monoclonal antibody
Detection Antibody:	30 mL of biotinylated anti-mouse IFN-γ antibody containing FBS* and ProClin™- 150 as preservative
Standards:	4 vials lyophilized recombinant mouse IFN-γ
Enzyme Concentrate (250×):	150 µL of 250× concentrated Streptavidin-horseradish peroxidase conjugate with BSA* and ProClin™- 150 as preservative
Standard/Sample Diluent:	30 mL of animal serum* with 0.09% sodium azide as preservative
ELISA Diluent:	12 mL of a buffered protein base with 0.09% sodium azide as preservative
Wash Concentrate (20×):	100 mL of 20× concentrated detergent solution with ProClin™- 150 as preservative
TMB One-Step Substrate Reagent:	30 mL of 3,3',5,5'-tetramethylbenzidine (TMB) in buffered solution
Stop Solution:	13 mL of 1 M phosphoric acid
Plate Sealers:	4 sheets with adhesive backing

**Source of all serum proteins is from USDA inspected abattoirs located in the United States*

Materials Required but not Provided

- Microplate reader capable of measuring absorbance at 450 nm
- Precision pipettes to deliver 50 µL and 100 µL volumes
- Adjustable 1 mL, 5 mL, 10 mL, 25 mL pipettes for reagent preparation
- Deionized or distilled water
- Wash bottle or automated microplate washer
- Log-log graph paper or computer and software for ELISA data analysis
- Tubes to prepare standard dilutions
- Laboratory timer
- Absorbent paper

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Storage Information

1. Store unopened kit at 2 - 8°C. Do not use kit after expiration date.
2. Before use, bring all reagents to room temperature (18 - 25°C). Immediately after use, return to proper storage conditions.
3. Lyophilized standards are stable until kit expiration date. After reconstitution, use freshly reconstituted standard within 12 hours (stored at 2 - 8°C).

Warnings and Precautions

1. Reagents that contain preservatives may be toxic if ingested, inhaled, or come in contact with skin.
2. Avoid contact of skin, eyes, or clothing with Stop Solution or Substrate Reagents.
3. Handle all serum and plasma specimens in accordance with NCCLS guidelines for preventing transmission of blood-borne infections.
4. Standard/Sample Diluent and ELISA Diluent contain 0.09% sodium azide. Sodium azide yields highly toxic hydrazoic acid under acidic conditions. Dilute azide compounds in running water before discarding to avoid accumulation of potentially explosive deposits in plumbing.
5. ***Warning***

   Wash Concentrate (20X) (component 51-9003738) contains 0.002% (w/w), Mouse IFN-gamma Lyophilized Standard (component 51-9004471) contains 0.03% (w/w) and Detection Antibody Biotin Anti-Mouse IFN-gamma (component 51-27192E) contains 0.003% (w/w) of a CMIT/MIT mixture (3:1), which is a mixture of: 5-chloro-2-methyl-4-isothiazolin-3-one [EC No 247-500-7] and 2-methyl-4-isothiazolin-3-one [EC No 220-239-6] (3:1).

   Hazard statements

   May cause an allergic skin reaction.

   Precautionary statements

   Wear protective gloves / eye protection.

   Wear protective clothing.

   Avoid breathing mist/vapours/spray.

   If skin irritation or rash occurs: Get medical advice/attention.

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IF ON SKIN: Wash with plenty of water.

Dispose of contents/container in accordance with local/regional/national/international regulations.

6. ***Danger***

Stop Solution (component 51-2608KC) contains 15.23% phosphoric acid (w/w).

Hazard statements

Causes severe skin burns and eye damage.

Precautionary statements

Wear protective gloves / eye protection.

Wear protective clothing.

IF ON SKIN (or hair): Remove/Take off immediately all contaminated clothing. Rinse skin with water/shower.

IF IN EYES: Rinse cautiously with water for several minutes. Remove contact lenses, if present and easy to do.

Continue rinsing.

IF INHALED: Remove victim to fresh air and keep at rest in a position comfortable for breathing.

Dispose of contents/container in accordance with local/regional/national/international regulations.

Specimen Collection and Handling

Specimens should be clear, non-hemolyzed and non-lipemic. Specimens with expected values greater than 200 pg/mL should be diluted with Standard/Sample Diluent prior to running the assay.

Cell culture supernatants: Remove any particulate material by centrifugation and assay immediately or store samples at ≤ -20°C. Avoid repeated freeze-thaw cycles.

Serum: Use a serum separator tube (eg, BD Vacutainer® Cat. No. 366430) and allow samples to clot for 30 minutes, then centrifuge for 10 minutes at 1000 × *g*. Remove serum and assay immediately or store samples at ≤ -20°C. Avoid repeated freeze-thaw cycles.

Plasma: Collect plasma using heparin, EDTA, or citrate as anticoagulant. Centrifuge for 10 minutes at 1000 × *g* within 30 minutes of collection. Assay immediately or store samples at ≤ -20°C. Avoid repeated freeze-thaw cycles.

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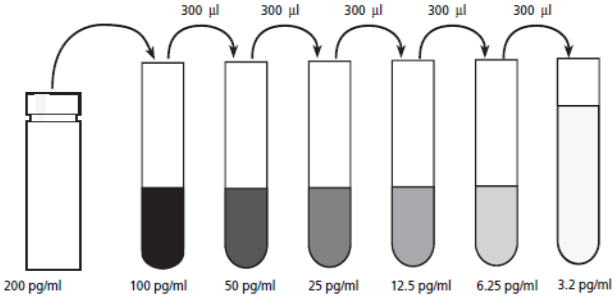
Reagent Preparation

1. Bring all reagents to room temperature (18 - 25°C) before use.
2. Standards
   a. Reconstitute 1 vial lyophilized Standard with required volume (noted on vial label) of Standard/Sample Diluent to prepare a 200 pg/mL stock standard. Allow the standard to equilibrate for at least 15 minutes before making dilutions. Gently vortex to mix.
   b. Add 300 µL Standard/Sample Diluent to 6 tubes. Label as 100 pg/mL, 50 pg/mL, 25 pg/mL, 12.5 pg/mL, 6.25 pg/mL, and 3.2 pg/mL.
   c. Perform serial dilutions by adding 300 µL of each standard to the next tube and vortexing between each transfer. The undiluted standard serves as the high standard (200 pg/mL). The Standard/ Sample Diluent serves as the zero standard (0 pg/mL).



3. Working Detector

   Note: See ***Assay Procedure***, step 5.

4. Wash Buffer

   Note: If the Wash Concentrate contains visible crystals, warm to room temperature and mix gently until dissolved. Dilute required quantity of 20× Wash Concentrate with deionized or distilled water, mix. (To prepare 2,000 mL, add 100 mL Wash Concentrate to 1,900 mL water. At least 500 mL solution should be prepared for a full 96-well plate).

5. TMB One-Step Substrate Reagent

   Add required volume of TMB One-Step Substrate Reagent to a clean tube or reservoir. Pipette out from the tube/ reservoir instead of directly from the bottle, to prevent contamination. Avoid prolonged exposure to light or contact with metal, air, or extreme temperature as color may develop.

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Assay Procedure

1. Bring all reagents and samples to room temperature (18 - 25°C) prior to use. It is recommended that all standards and samples be run in duplicate. A standard curve is required in each assay run.
2. Remove required quantity of test strips/wells, place in well holder.

   Note: Wells are provided in breakable 8-well strips. Strips may be "broken" into individual wells, replaced in well holder, and assayed. Return any unused wells to sealed pouch for 2 - 8°C storage.
3. Pipette 50 µL of ELISA Diluent into each well.
4. Pipette 50 µL of each standard (see ***Reagent Preparation***, step 2) and sample into appropriate wells. Gently shake/tap the plate to mix. Cover wells with Plate Sealer and incubate for 2 hours at room temperature.
5. Prepare Working Detector. Within 15 minutes prior to use, pipette required volume of Detection Antibody into a clean tube or flask. Add required quantity of Enzyme Concentrate (250×), vortex or mix well. For a full 96-well plate, add 48 µL of Enzyme Concentrate into 12 mL of Detection Antibody.
6. Decant or aspirate contents of wells. Wash wells by filling with at least 300 µL/well prepared Wash Buffer (see ***Reagent Preparation***, step 4) and then decanting/aspirating. Repeat wash 4 times for a total of 5 washes. After the last wash, blot plate on absorbent paper to remove any residual buffer. Complete removal of liquid is required for proper performance.
7. Add 100 µL of prepared Working Detector (see ***step 5*** above) to each well. Cover wells with Plate Sealer and incubate for 1 hour at room temperature.
8. Wash wells as in Step 6, but a total of 7 times.

   Note: In this final wash step, soak wells in wash buffer for 30 seconds to 1 minute for each wash. Thorough washing at this step is very important.
9. Add 100 µL of TMB One-Step Substrate Reagent to each well. Incubate plate (without Plate Sealer) for 30 minutes at room temperature in the dark.
10. Add 50 µL of Stop Solution to each well.
11. Read absorbance at 450 nm within 30 minutes of stopping reaction. If wavelength correction is available, subtract the optical density readings at 570 nm from readings at 450 nm.

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Assay Procedure Summary

1. Add 50 µL ELISA Diluent to each well.
2. Add 50 µL standard or sample to each well.

   Incubate 2 hours at room temperature.
3. Aspirate and wash 5 times.
4. Add 100 µL prepared Working Detector to each well.

   Incubate 1 hour at room temperature.
5. Aspirate and wash/soak 7 times.
6. Add 100 µL Substrate Reagent to each well.

   Incubate 30 minutes at room temperature.
7. Add 50 µL Stop Solution to each well.

   Read at 450 nm within 30 minutes.

   λ correction 570 nm.

Calculation of Results

Calculate the mean absorbance for each set of duplicate standards, controls and samples. Subtract the mean zero standard absorbance (ie, plate background) from each.

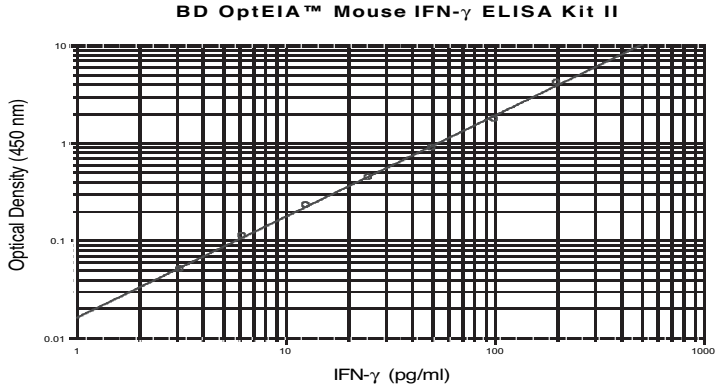
Plot the standard curve on log-log graph paper, with IFN-γ concentration on the x-axis and absorbance on the y-axis. Draw the best fit straight line through the standard points.

To determine the IFN-γ concentration of the unknowns, find the unknowns' mean absorbance value on the y-axis and draw a horizontal line to the standard curve. At the point of intersection, draw a vertical line to the x-axis and read the IFN-γ concentration. If samples were diluted, multiply the interpolated IFN-γ concentration by the dilution factor.

Computer-based curve-fitting statistical software may also be employed.

Typical Data

This standard curve is for demonstration only. A standard curve must be run with each assay.



Concentration (pg/mL)	OD1	OD2	Mean	Zero Standard Subtracted
0	0.045	0.045	0.045	0.000
3.125	0.098	0.095	0.097	0.052
6.25	0.166	0.151	0.159	0.114
12.5	0.285	0.269	0.277	0.232
25	0.493	0.485	0.489	0.444
50	0.942	0.897	0.920	0.875
100	1.705	1.733	1.719	1.674
200	4.200	4.200	4.200	4.155

Limitations of the Procedure

1. This kit is intended for use as an integral unit. Do not mix reagents from different kit lots. Reagents from other manufacturers/other available clones should not be used in this kit.
2. Interference by drug metabolites, soluble receptors, or other binding proteins in specimens has not been thoroughly investigated. The possibility of interference cannot be excluded.

Performance Characteristics

Sensitivity

The minimum detectable dose of IFN-γ was determined to be 0.762 pg/mL. This is defined as two standard deviations above the mean optical density of 20 replicates of the zero standard.

Recovery

Four different amounts of IFN-γ were spiked into pooled mouse sera and cell culture media samples. Results are compared with the same amounts of IFN-γ spiked into Standard/Sample Diluent, as follows:

	Spike Concentration (pg/mL)	Average % Recovery	Range
Serum (4 serum samples from 4 different commercial sources)	100 50 25 12.5	100.2 ± 4.39 100.9 ± 4.25 104.6 ± 4.65 106.4 ± 13.43	96.4 - 107.4 94.8 - 105.4 96.7 - 108.5 92.5 - 128.6
Cell culture media (4 samples)	100 50 25 12.5	99.2 ± 1.96 93.0 ± 3.79 87.5 ± 4.28 84.2 ± 8.12	97.5 - 102.3 88.6 - 98.0 81.5 - 91.6 74.8 - 96.6

Linearity

Four pooled mouse sera samples and four cell culture media samples were spiked with recombinant IFN-γ, serially diluted with Standard/Sample Diluent, and run in the BD OptEIA™ Mouse IFN-γ ELISA Kit II. Results are as follows:

Dilution		Pooled Serum (n = 4)	Cell culture media (n = 3)
1:2	Average % of Expected Range	100.9 ± 5.60 93.7 - 109.4	106.3 ± 3.12 103.2 - 111.3
1:4	Average % of Expected Range	95.0 ± 4.00 89.6 - 100.9	105.1 ± 4.50 99.3 - 111.9
1:8	Average % of Expected Range	88.0 ± 3.72 83.7 - 93.9	99.5 ± 3.58 94.6 - 104.7
1:16	Average % of Expected Range	86.2 ± 2.84 83.7 - 91.0	93.8 ± 4.79 88.6 - 98.8
1:32	Average % of Expected Range	78.3 ± 2.2.20 75.8 - 81.0	82.1 ± 7.21 72.7 - 92.7

Specificity

Cross-Reactivity: The following factors were tested in the BD OptEIA™ Mouse IFN-γ ELISA Kit II assay at ≥ 10 ng/mL and no cross-reactivity was identified.

Recombinant Human

IFN-γ

Recombinant Mouse

IL-1α, IL-1β, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-10, IL-12 (p70), TNF, and TNFRII

Recombinant Rat

IFN-γ (10 ng/mL)

Recombinant Pig

IFN-γ (4 ng/mL)

Precision

Intra-assay

Twenty-four replicates each of three different levels of IFN-γ were tested in one plate. The following results were observed:

Number of Replicates	24	24	24
Mean Concentration	94.9 pg/mL	49.4 pg/mL	25.9 pg/mL
SD	2.57	1.71	0.99
%CV	2.7	3.5	3.8

Inter-assay

Three different levels of IFN-γ were tested in four different plates. The following results were observed:

Number of Replicates	32	32	32
Mean Concentration	95.4 pg/mL	49.2 pg/mL	25.8 pg/mL
SD	5.91	2.30	1.65
%CV	6.19	4.67	6.39

Standardization

The immunoassay is calibrated against recombinant mouse IFN-γ.

Expected Values

Serum/Plasma

Individual mouse serum samples from twenty three apparently healthy normal mice were tested in this assay. All the 23 samples measured less than 3.125 pg/mL (lowest standard level).

Cell Culture Supernatants

Mouse bone marrow cells and splenocytes from apparently healthy, normal NZB and C57BL/6 mice were cultured in RPMI 1640 complete medium with 10% fetal bovine serum at 1×10^6 cells/mL, and stimulated with PMA/ionomycin for 24 hours. Culture supernatants were collected and quantified for IFN-γ using a BD OptEIA™ Mouse IFN-γ ELISA Kit II. The results are as follows:

Mouse No.	(ng/mL)	Cell Source	Cell Treatments
1	1656.5 pg/mL	NZB	P + I for 24hr
2	1618.3 pg/mL	NZB	P + I for 24hr
3	1808.5 pg/mL	NZB	P + I for 24hr
4	1457.5 pg/mL	NZB	P + I for 24hr
5	7716.5 pg/mL	C57	P + I for 24hr
6	16345.5 pg/mL	C57	P + I for 24hr

Troubleshooting

Problem	Possible Source	Corrective Action
Poor Precision	• Inadequate washing / aspiration of wells • Inadequate mixing of reagents • Imprecise / inaccurate pipetting • Imprecise sealing of plate	• Check function of washing system • Ensure adequate mixing • Check / calibrate pipettes • Ensure complete sealing of plate
Poor Standard Curve	• Improper standard handling / dilution • Incomplete washing / aspiration of wells • Imprecise / inaccurate pipetting	• Ensure correct preparation of standards • Check function of washing system • Check / calibrate pipettes
Low Signal	• Inadequate reagent volumes added to wells • Incorrect incubation times / temperature • Overly high wash / aspiration pressure from automated plate-washer.	• Check / calibrate pipettes • Ensure sufficient incubation times / reagents warmed to room temperature • Utilize manual washing

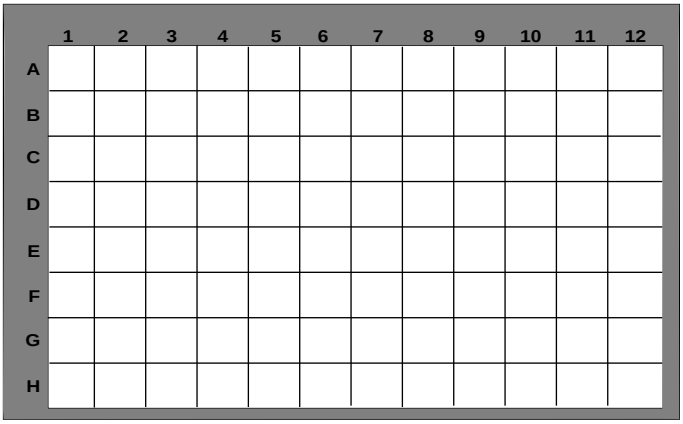
References

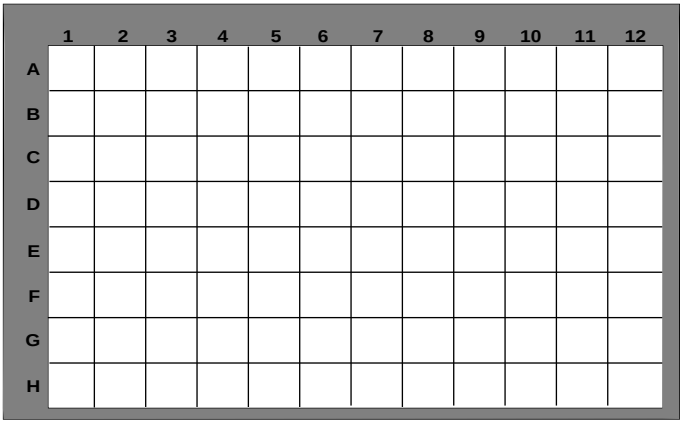
1. Gray, P.W., and D.V. Goeddel. 1983. Cloning and expression of murine immune interferon cDNA. *Proc. Natl. Acad. Sci USA.* 80: 5842 - 5846.

2. Farrar, M.A., and R.D. Schreiber. 1993. The molecular cell biology of interferon-γ and its receptor. *Annu. Rev. Immunol.* 11: 571-611.

3. Green, J.A., T.J. Yeh, and J.C. Overall, Jr. 1980. Rapid, quantitative, semiautomated assay for virus-induced and immune human interferons. *J. Clin. Microbiol.* 12: 433 - 438.

Plate Templates





Notes

Notes

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