# Human IFN-γ FCM Kit

# (Flow Cytometry Assay)

Catalog Number：CFA-C001

Pack Size: 96 tests

**IMPORTANT: Please carefully read this manual before performing your experiment.**

**<u>For Research Use Only. Not For Use in Diagnostic or Therapeutic Procedures</u>**

## INTENDED USE

The kit is developed for quantitative detection of IFN- γ in human serum and cell culture supernatants. It is intended for research use only (RUO).

## BACKGROUND

Human Interferon-γ (IFN-γ) is a potent multifunctional cytokine which is secreted primarily by activated NK cells and T cells. Originally characterized based on its anti-viral activities, IFN-γ also exerts anti-proliferative, immunoregulatory, and proinflammatory activities. IFN-γ can upregulate MHC class I and II antigen expression by antigen-presenting cells. Natural IFN-γ is a dimeric protein with subunits of 143 amino acids. IFN-γ is synthesized as a precursor protein of 166 amino acids including a secretory signal sequence of 23 amino acids. The protein is glycosylated.

## PRINCIPLE OF THE ASSAY

Human IFN- γ FCM Kit (Flow Cytometry Assay) gives a quantitatively result of IFN-γ. The performance of this kit has been optimized for specific analysis of IFN-γ in cell culture supernatants, plasma and serum samples. The kit provides sufficient reagents for the quantitative analysis of 96 tests.

The antibody encapsulated 1-plex beads, protein (IFN-γ) in specimens and biotin-conjugated detection antibody formed a sandwich complex as bead-analyte-detection antibody. SA-PE was added in and react with biotinylated detection antibody, the intensity of PE fluorescence, in proportion to the recombinant protein titer in specimens, was assessed by flow cytometry at wavelength of 575 nm approximately. The intensity of APC fluorescence was applied to classify bead populations, at wavelength of 670 nm approximately.

## PRECAUTIONS

1. This kit is for research use only and is not for use in diagnostic or therapeutic applications.
2. The kit is suitable for cell supernatant, serum and plasma samples.
3. Do not use reagents past their expiration date.
4. Do not mix or substitute reagents with those from other kits or other lot number kits.



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5. If samples generate values higher than the highest standard, dilute the samples with the appropriate calibrator diluent and repeat the assay. If cell supernatant samples need step dilution, except for the final dilution with diluent, other intermediate dilutions can be in cell culture medium.
6. Differences in test results can be caused by a variety of factors, including laboratory operator, pipette usage, plate washing technique, reaction time or temperature, and kit storage.
7. This kit is designed to remove or reduce some endogenous interference factors in biological samples, and not all possible influencing factors have been removed.

## MATERIALS PROVIDED

**Table1. Materials provided**

| Catalog | Components | Size (96 tests) | Format | Storage | |
|---|---|---|---|---|---|
| | | | | Unopened | Opened |
| CFA001-C01 | Calibrator | 20 µg | Powder | 2-8 °C | -20 °C |
| CFA001-C02 | IFN-γ Beads | 96 tests | Beads suspension | 2-8 °C Light-sensitive | 2-8 °C Light-sensitive |
| CFA001-C03 | 2 × Assay Buffer | 40 mL | Liquid | 2-8 °C | 2-8 °C |
| CFA001-C04 | 10× Wash Buffer | 10 mL | Liquid | 2-8 °C | 2-8 °C |
| CFA001-C05 | Detection antibody | 1 mL | Liquid | 2-8 °C | 2-8 °C |
| CFA001-C06 | SA-PE | 40 µg | Powder | 2-8 °C Light-sensitive | 2-8 °C Light-sensitive |
| CFA001-C08 | 96-well Sealing film | 2 pieces | / | 2-8 °C or RT | RT |
| CFA001-C09 | APC Positive Control | 500 µL | Beads suspension | 2-8 °C Light-sensitive | 2-8 °C Light-sensitive |
| CFA001-C10 | PE Positive Control | 500 µL | Beads suspension | 2-8 °C Light-sensitive | 2-8 °C Light-sensitive |

## KIT STORAGE AND EXPIRATION DATE

1. The unopened kit is stable for 18 months from the date of manufacture if stored at 2°C to 8°C.
2. The opened kit should be stored per Table 1. The shelf life is 30 days from the date of opening.

***Note:*** *a. Do not use reagents past their expiration date.*

*b. Find the expiration date on the outside packaging.*

## REAGENTS/EQUIPMENT NEEDED BUT NOT SUPPLIED

1. Single-channel pipettes, multi-channel pipettes and pipette tips
2. Reagent reservoirs for multichannel pipette
3. Polypropylene microcentrifuge tubes for samples collection or dilution
4. Deionized or distilled ultrapure water
5. 96-well magnetic separator
6. Horizontal orbital shaker for 96-well plate
7. Vortex mixer
8. Flow cytometer equipped with two lasers:

   (1) Excitation at 488 nm or 532 nm, emission around 575 nm;

   (2) Excitation around 633 nm, emission around 670 nm

## PREPARATIOM OF SAMPLES

1. Serum collection and storage

1.1 Fresh blood samples were obtained from venous, keep at room temperature for more than 30 minutes. After coagulation, 2, 000 g centrifuge for 10 minutes at 4 ℃ (Excessive centrifugation might lead to hemolysis). Aspirate the serum layer and avoid the contamination of blood cells.

1.2 Serum layer were centrifuged 16, 000 g for 10 minutes at 4 ℃. Discard the precipitates and the supernatant was the blood serum freshly prepared.

1.3 Use the serum immediately or keep at -80 ℃ for long time storage.

2. Plasma collection and storage

2.1 Fresh blood samples are obtained from venous, adding anticoagulant sodium citrate, such as EDTA or heparin. After coagulation, 2, 000 g centrifuge for 10 minutes at 4 ℃. Carefully aspirate the plasma

layer, and avoid the contamination with blood cells.

2.2 Centrifuge the plasma layer 16, 000 g for 10 minutes at 4 ℃. Discard the precipitates, and keep supernatant as the freshly prepared plasma. Use the plasma immediately or storage at -80 ℃.

***Note 1:*** *Frozen serum or plasma should be mixed thoroughly after thawing, and remove all visible debris by centrifuge. Thawed serum or plasma must be used up and avoid repeated freeze-thaw cycles.*

***Note 2:*** *Hemolyzed, icteric and lipemic samples are not validated for use in this assay.*

3. Preparation of Reagents and Buffer

3.1 Beads suspension working solution

Beads from the kit (from ID# CFA001-C02). Vortex beads suspension vigorously no less than 30 seconds. Immediately transfer required volume of beads to a microcentrifuge tube and mix with required volume of Assay Buffer.

***Note:*** *DO NOT aspirate beads suspension less than 20 μL to minimize pipetting errors. Perform a serial dilution if only a few beads needed for the assay.*

To setup ONE test in ONE well in 96-well V-bottom plate, add beads suspensions for 1-plex, then replenish with Assay Buffer to total volume of 120 μL, as indicated in Table 2.

**Table 2. Preparation of beads suspension working solution for one test**

| 1-plex | Single Capture Coating Beads, ID# FCM05-C02 | Add Assay Buffer |
|---|---|---|
| 1-plex | 1 μL | 119 μL |

3.2 Detection Antibody working solution

Bring Detection Antibody to room temperature. Aspirate 1 mL Detection Antibody, mixed with 11 mL Assay Buffer.

3.3 Assay Buffer

Bring 2 × Assay Buffer to room temperature. Aspirate 40 mL 2 × Assay Buffer, mixed with 40 mL deionized water.

3.4 Wash Buffer

Bring 10 × Wash Buffer to room temperature. Aspirate 10 mL 10× Wash Buffer, mixed with 90 mL deionized water.

3.5 SA-PE working solution



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Reconstitute lyophilized SA-PE powder in 66.7 μL deionized water with an initial concentration of 600 μg/mL. To dissolve completely we recommend sit the bottle at room temperature for 15 minutes. Aspirate 55 μL in a new 50 mL tube, added to 22 mL with Assay Buffer and the solution is SA-PE working solution.

3.6 Preparation of Calibrator

3.6.1 Reconstitute lyophilized calibrator powder in 1000 μL deionized water, as calibrator stock#1 with each analyte 20 μg/mL respectively. For completely dissolving, keep the bottle at room temperature at least for 15 minutes.

***Note:*** *Mix or reconstitute protein reagent gently, avoid bubbles and foam.*

3.6.2 For multiplex assay, aspirate 50 uL calibrator Cm1 in a new tube, add in Assay Buffer to 950 μL, labeled as calibrator Cm2. The concentration of each analyte is 1 μg/mL respectively.

3.6.3 A new microcentrifuge tube, add in 900 μL Assay Buffer, 100 μL calibrator stock#2, mix well, and labeled as Cm3.

3.6.4 Repeat operation of step 3.6.3 with Cm3, mix well, and labeled as C11.

3.6.5 Performing 2-fold serial dilutions from C11, add 500 μL Assay Buffer, labeled as C10, C9, C8, C7, C6, C5, C4, C3, C2 and C1 respectively, as shown in Table 3.

***Note:*** *Mix thoroughly before making the next dilution.*

**Table 3. Preparation of Calibrator**

| Calibrator ID | Serial Dilution | Assay Buffe add in (μL) | Calibrator add in (μL) | Final Concentration (pg/mL) |
|---|---|---|---|---|
| Cm2 | 20 | 950 | 50 μL of Cm1 | 1,000,000 |
| Cm3 | 10 | 900 | 100 μL of Cm2 | 100,000 |
| C11 | 10 | 900 | 100 μL of Cm3 | 10,000 |
| C10 | 2 | 500 | 500 μL of C11 | 5,000 |
| C9 | 2 | 500 | 500 μL of C10 | 2,500 |
| C8 | 2 | 500 | 500 μL of C9 | 1250 |
| C7 | 2 | 500 | 500 μL of C8 | 625 |
| C6 | 2 | 500 | 500 μL of C7 | 312.5 |
| C5 | 2 | 500 | 500μL of C6 | 156.3 |
| C4 | 2 | 500 | 500 μL of C5 | 78.1 |



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| C3 | 2 | 500 | 500 μL of C4 | 39.1 |
|---|---|---|---|---|
| C2 | 2 | 500 | 500 μL of C3 | 19.5 |
| C1 | 2 | 500 | 500 μL of C2 | 9.8 |
| C0 | - | 500 | - | 0 |

## ASSAY PROCEDURE

1. Plasma/serum preparation (one sample repeat). Dilute 30 μL freshly prepared plasma or thawed serum with 60 μL Assay Buffer, mix homogenously and ready for being used.
2. Add serial dilutions of calibrator or samples to 96-well V-bottom plate, 30 μL per well.

*Note: Run calibrators in duplicates. Follow the attached Plate Layout to achieve good accuracy.*

3. Add beads suspension working solution to 96-well V-bottom plate, 120 μL per well.
4. Add detection antibody working solution, 100 μL per well.
5. Seal the plate. Incubate at room temperature for 120 minutes, with continuous shaking 400-600rpm to ensure the beads always suspended homogenously in the solution. Avoid light.
6. Place the 96-well V-bottom plate onto the magnetic separation rack for 2 minutes before discarding supernatant.

***Note:** Magnet varies in strength. It may take a few seconds to minutes, to complete the separation.*

7. Remove plate from separation rack and reconstitute each well in 200 μL of Wash Buffer. Mix thoroughly using pipette by aspirating and dispensing 2-3 times. Place the 96-well V-bottom plate onto the magnetic separation rack for 2 minutes before discarding supernatant.
8. Place the 96-well V-bottom plate onto the magnetic separation rack for 2 minutes before discarding supernatant. Repeat step 2.7.
9. Remove the plate from the magnet. Add SA-PE working solution, 200 μL per well.
10. Seal the plate. Incubate at room temperature for 30 minutes, with continuous shaking to ensure the beads always suspended homogenously in the solution. Avoid light.
11. Place the 96-well V-bottom plate onto the magnetic separation rack for 2 minutes before discarding supernatant. Repeat step 2.7.
12. Add 150 μL Wash Buffer to each well. Mix by pipetting up and down. Ensure the beads well separated and not aggregated.
13. Subject to flow cytometry analysis. If not being analyzed immediately, store at 2~8 ℃ and avoided light. Flow cytometry assay should be performed within 2 hours.

***Note**: Resuspend beads immediately prior to reading by pipetting up and down.*

## FLOW CYTOMETER SETUP

1. Flow cytometer equipped with two lasers are compatible with the assay

    (1) excitation laser at 488 nm or 532 nm, and emission around 575 nm;

    (2) excitation laser around 633 nm, and emission around 670 nm.

   Instruments tested by this assay were represented in Table 4.

**Table 4. Partial list of compatible flow cytometers**

| Manufacturer | Verified instrument model | Classification Channel | Reporter Channel |
|---|---|---|---|
| BD Biosciences | BD FACSLyric™ | APC | PE |
| Beckman Coulter | Cytoflex S | R660-APC | Y585-PE |
| Thermo Fisher Scientific | Attune NxT | RL1 | YL1 |
| Luminex Corporation | Guava easy Cyte3L | RED-R | YEL-B |

2. Channel setup

2.1 PE Positive Control vortex 30 sec, subject to flow cytometry. For the voltage setup of the reporter channel, not as medium flow rate as samples running, we recommend a low rate and 8E5 as a threshold value for the PE signal.

2.2 APC Positive Control using for the setup of the classification channel as the PE Beads, the APC signal located at right range side of the detection platforms but not with an outside distribution is an optimal situation.

2.3 Select medium flow rate.

2.4 Sep up 500 events or beads per plex collected in P1 gate as stop criteria.

## DATA ACQUISITION AND ANALYSIS

3 Data acquisitionMake sure the flow cytometer is well tuned, following the instrument user guide and method configuration illustrated above.

3.2 Create an experiment in 96-well plate format.

*__Note:__ If 96-well plate loader is not available, transfer the samples to FACS tubes, replenish 100*

*μL Wash Buffer, and read one by one.*

3.3 Resuspend beads by pipetting up and down.

3.4 Load the plate and start acquisition.

3.5 Record median fluorescence intensity (MFI) of PE channel.

3.6 Data analysis

3.6.1 Two-log-linear fit curve model is applied by data analysis with GraphPad by plotting Log10 concentration value of serial diluted calibrators against median fluorescence intensity (MFI) of PE channel, or you can use the FCAP for data analysis. We recommend the $r^2$ value of the curve above 0.99.

3.6.2 Calculate the concentration of unknown from the calibration curve of each analyte.

3.6.3 Determine the concentration of blood specimens after multiplying by the dilution factor (3×).



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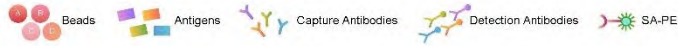
# QUICK GUILD



**1**

96-well plate
★ Add 30 μL calibrator or samples
★ Add 120 μL beads suspension working solution
★ Add 100 μL detection antibody working solution

Shake, avoid light

18-25℃ 120 minutes

Place the 96 well plate on magnetic separation rack and stay for 2 minutes. Discard supernatant.

**2**

★ Wash with 200 μL 1 × wash buffer, pipetting up and down for 2-3 times

Place the 96 well plate on magnetic separation rack and stay for 2 minutes. Discard supernatant.

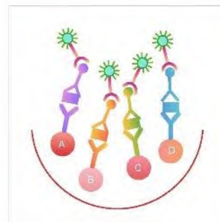
**3**

★ Add 200 μL SA-PE working solution

Shake, avoid light

18-25℃ 30 minutes

Place the 96 well plate on magnetic separation rack and stay for 2 minutes. Discard supernatant.



**4**

★ Wash with 200 μL 1 × wash buffer, pipetting up and down for 2-3 times
★ Resuspend with 150 μL Dilution Buffer

**5**

★ Flow cytometry analysis. If not being analyzed immediately, store at 2~8℃, avoid light and should be performed within 2 hours.



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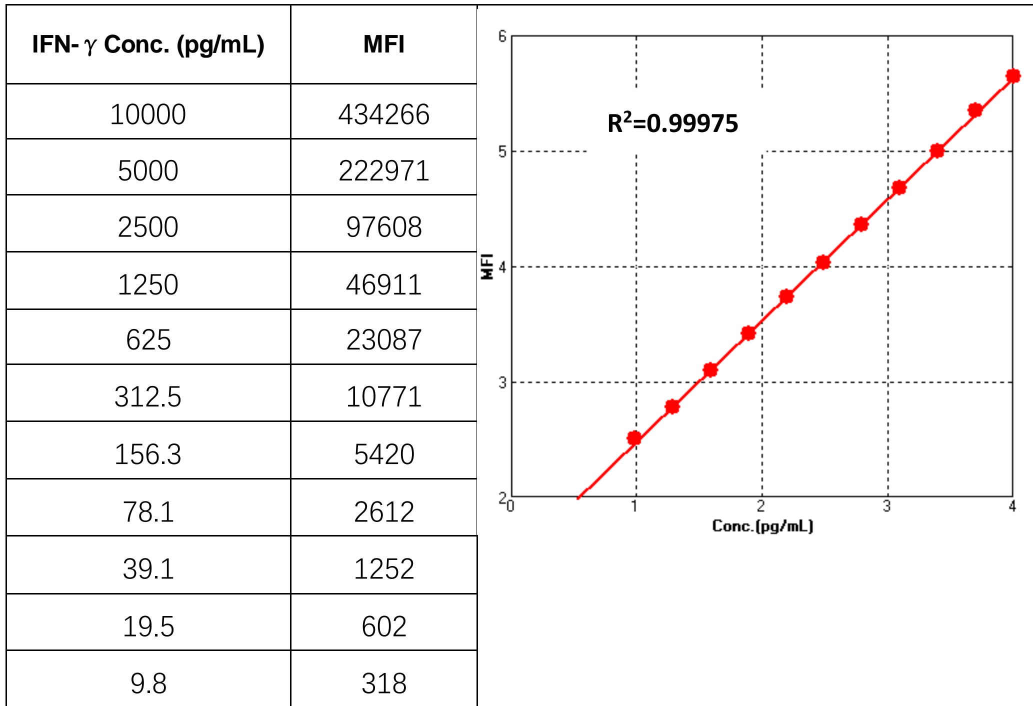
# TYPICAL DATA

The following data is for reference only. The sample concentration was calculated based on the results of the standard curve.



| IFN-γ Conc. (pg/mL) | MFI |
|---|---|
| 10000 | 434266 |
| 5000 | 222971 |
| 2500 | 97608 |
| 1250 | 46911 |
| 625 | 23087 |
| 312.5 | 10771 |
| 156.3 | 5420 |
| 78.1 | 2612 |
| 39.1 | 1252 |
| 19.5 | 602 |
| 9.8 | 318 |





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