Human IFN-γ Kit
(Flow Cytometry Bead 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, plasma and cell culture supernates. It is intended for research use only (RUO).

BACKGROUND

Human IFN-γ (Immune interferon, Type II interferon, T cell interferon, Macrophage-activating factor (MAF), IFN-γ, IFN-gamma, etc.) 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. Flow Cytometry Assay provide a method of capturing a soluble analyte or set of analytes with beads of known size and fluorescence, making it possible to detect analytes using flow cytometry.

PRINCIPLE OF THE ASSAY

Human IFN-γ FCM Kit (Flow Cytometry Assay) gives a quantitatively result of Interferon-γ (IFN-γ). The performance of this kit has been optimized for specific analysis of Interferon-γ (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.

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4. Do not mix or substitute reagents with those from other kits or other lot number kits.
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-C07	96-well V-bottom plate	1 plate	/	2-8 °C or RT	RT
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. Unopened: Store at 2℃~8℃ upon receipt. The expiration date is labeled on the package box. DO NOT use reagents beyond expiration date.
2. Opened: The opened kit should be stored per component, as indicated in Table 1. The shelf life of all components and dilution components are 30 days from the date of opening.

Note: *Freeze and thaw NO MORE THAN 2 times, once calibrator (ID# CFA001-C01) is reconstituted.*

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. Cell culture medium

1.1 Fresh collected or -80℃ stored medium centrifuge 3, 000 g 10 minutes at 4 °C, aspirate the supernatant and used for the next assay or storage at -80 ℃.

2. Serum collection and storage

2.1 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 °C (Excessive centrifugation might lead to hemolysis). Aspirate the serum layer and avoid the contamination of blood cells.

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

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

3. Plasma collection and storage

3.1 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.

3.2 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.*

4. Preparation of Reagents and Buffer

4.1 Assay Buffer

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

4.2 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# CFA001-C02	Add Assay Buffer
1-plex	1 μL	119 μL

4.3 Detection Antibody working solution

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

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4.4 Wash Buffer

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

4.5 SA-PE working solution

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.

5. Preparation of Calibrator

5.1 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*.

5.2 For multiplex assay, aspirate 50 uL calibrator stock#1 in a new tube, add in Assay Buffer to 950 µL, labeled as calibrator stock#2. The concentration of each analyte is 1 µg/mL respectively.

5.3 A new microcentrifuge tube, add in 900 µL Assay Buffer, 100 µL calibrator stock#2, mix well, and labeled as stock#3

5.4 Repeat operation of step 5.3 with stock#3, mix well, and labeled as C11.

5.5 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*.

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Table 3. Preparation of Calibrator

Calibrator ID	Serial Dilution	Assay Buffe add in (μL)	Calibrator add in (μL)	Final Concentration (pg/mL)
stock#2	20	950	50 μL of stock#1	1,000,000
stock#3	10	900	100 μL of stock#2	100,000
C11	10	900	100 μL of stock#3	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
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. Repeat the wash step above for two times.

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8. Remove the plate from the magnet. Add SA-PE working solution, 200 µL per well.

9. 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.

10. Place the 96-well V-bottom plate onto the magnetic separation rack for 2 minutes before discarding supernatant. Repeat step 7.

11. Add 150 µL Wash Buffer to each well. Mix by pipetting up and down. Ensure the beads well separated and not aggregated.

12. 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

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situation.

3. Select medium flow rate.

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

DATA ACQUISITION AND ANALYSIS

1. Data acquisition

1.1 Make sure the flow cytometer is well tuned, following the instrument user guide and method configuration illustrated above.

1.2 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.*

1.3 Resuspend beads by pipetting up and down.

1.4 Load the plate and start acquisition.

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

2. Data analysis

2.1 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.

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

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

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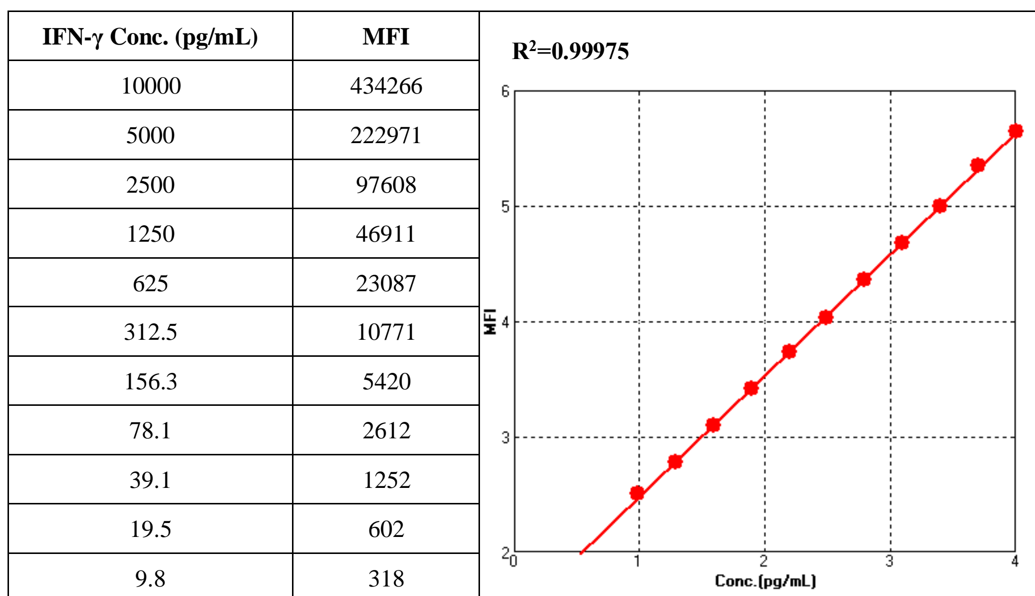
Typical data

For each experiment, a calibration curve needs to be set for each microplate, and the specific MFI value may vary depending on different laboratories, testers, or equipment. The following example data is for reference only. The sample concentration was calculated based on the results of the calibration 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

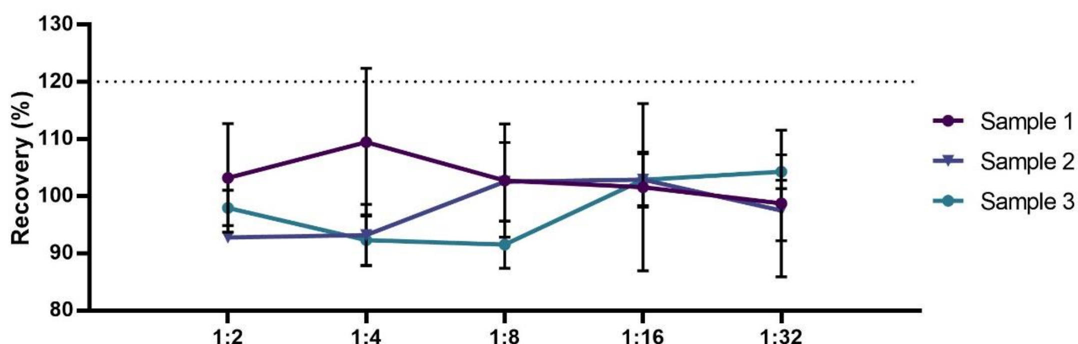
PERFORMANCE CHARACTERISTICS

1. Sensitivity

The minimum detectable concentration (MDC) of IFN-γ is typically less than 2.0 pg/mL. The MDC was determined by adding two standard deviations to the mean optical density value of twenty zero standard replicates and calculating the corresponding concentration.

2. Linearity

Three samples (Serum) spiked with high concentrations of 15000 pg/mL, 14000 pg/mL and 12000 pg/mL were serially diluted with dilution buffer to produce samples with values within the dynamic range of the assay and then assayed. The average recovery of IFN-γ for serum samples is 103.12%.



3. Intra-Assay Precision

Ten replicates of each of 4 samples containing different IFN- γ concentrations were tested in one assay. Acceptable criteria: CV＜10%.

Sample Concentration (pg/mL)	Mean (pg/mL)	SD	Numbers	CV
10000	10274.2	374.0	10	3.64%
5000	4778.0	329.7	10	6.90%
19.5	18.6	1.3	10	7.07%
9.8	9.9	1.0	10	9.89%

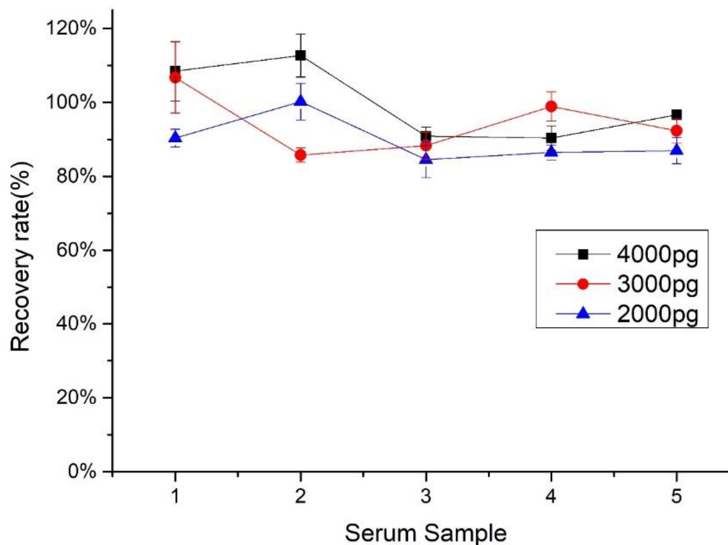
4. Inter-Assay Precision

Four samples containing different concentrations of IFN- γ were tested in independent assays. Acceptable criteria: CV<15%.

Sample Concentration (pg/mL)	Mean (pg/mL)	SD	Numbers	CV
10000	10323.6	472.8	10	4.58%
5000	5133.4	461.6	10	8.99%
19.5	18.9	1.8	10	9.27%
9.8	9.9	1.3	10	12.77%

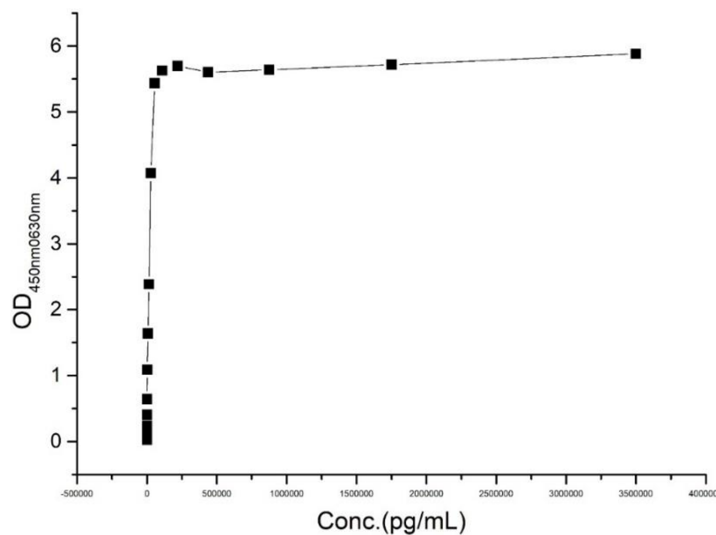
5. Recovery

Recombinant IFN-γ was spiked into 5 human serum samples, and then analyzed. The average recovery of IFN-γ for serum samples is 94.64%.



6. Hook Effect

Not be affected by IFN-γ concentrations up to 250 ng/mL.



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7. Specificity

No cross-reactivity was observed when this kit was used to analyze the following recombinant cytokines at up to 1 µg/mL.

Human			
IL-1β	IL-5	IL-8	MCP-1
IL-2	IL-6	IL-12p70	M-CSF
IL-4	IL-7	IL-10	TNF-α

8. CALIBRATION

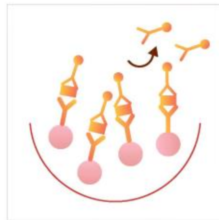
This immunoassay is calibrated against highly purified recombinant human IFN-γ produced at ACROBiosystems. The NIBSC/WHO International Standard for IFN-γ (82/587), which was intended as a potency standard, was evaluated in this kit.

QUICK GUILD

1
96-well plat
★ 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 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. Repeat the wash step above for two times.

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. Repeat the wash step above for two times.

4
★ Add 150 μL Wash Buffer to each well. Mix by pipetting up and down.

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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TROUBLE SHOOTING

Problem	Cause	Solution
Poor standard curve	* Inaccurate pipetting	* Check pipettes
Large CV	* Inaccurate pipetting * Air bubbles in wells	* Check pipettes * Remove bubbles in wells
High background	* Plate is insufficiently washed * Contaminated wash buffer	* Review the manual for proper wash. * Make fresh wash buffer
Very low readings across the plate	* Incorrect wavelengths * Insufficient development time	* Check filters/reader * Increase development time
Samples are reading too high, but standard curve looks fine	* Samples contain cytokine levels above assay range	* Dilute samples and run again
Drift	* Interrupted assay set-up * Reagents not at room temperature	* Assay set-up should be continuous - have all standards and samples prepared appropriately before commencement of the assay * Ensure that all reagents are at room temperature before pipetting into the wells unless otherwise instructed in the antibody inserts

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