Human Th1/Th2 Cytokines 6-Plex Panel 1

Catalog Number：FCMh-021

Assay Tests: 100 tests

For research use only, not for use in diagnostic or therapeutic procedures.

Please read this manual carefully before performing the experiment.

Product information

Cytokines are key mediators of immune regulation, inflammatory responses, and tumor metastasis, and their quantification is widely applied in infectious and autoimmune disease diagnostics, tumor auxiliary diagnosis, disease monitoring, therapeutic guidance, and prognosis assessment. Multiplex bead-based flow cytometry enables simultaneous measurement of multiple analytes in a single well, reducing sample volume and reagent consumption, and is compatible with standard flow cytometers.

The Human Th1/Th2 Cytokines 6-Plex Panel 1 allows simultaneous quantification of **IL-2, IL-4, IL-6, IL-10, TNF-alpha, and IFN-gamma** from a single sample. As the upgraded version of FCM-C05R, it adopts a streamlined **one-step incubation protocol to improve workflow efficiency and assay accuracy.** The kit has been validated for cell culture supernatants, plasma, and serum, and contains sufficient reagents for 100 tests.

Assay principle

The assay is a bead-based sandwich immunoassay (Figure 1). Each distinct bead population — discriminated by size and internal APC fluorescence — is pre-coated with a target-specific capture antibody. During incubation, the capture antibody binds the corresponding cytokine; a PE-conjugated detection antibody is then added to form a bead–analyte–PE sandwich complex. Individual bead populations are resolved by their APC signal, and the median PE fluorescence intensity (MFI) is proportional to the analyte concentration, which is interpolated from a calibration curve.

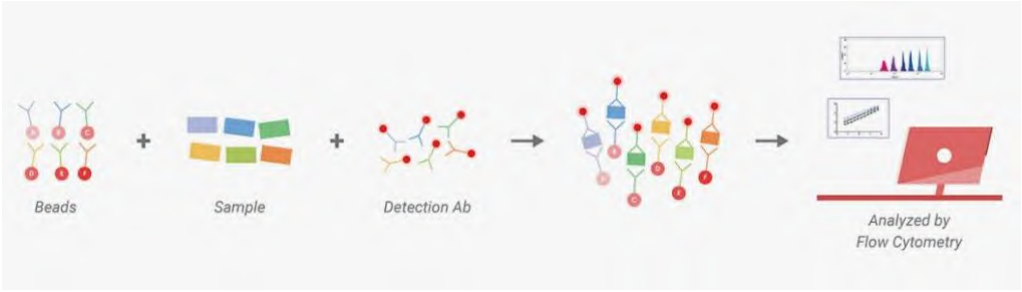


Figure 1 Assay Principle

NOTE:

- For research use only, not for use in diagnostic or therapeutic procedures.
- Please follow the instructions strictly, for optimal and consistent data output.
- Protect beads suspension, detection antibody from light at all times to prevent photobleaching.
- DO NOT mix or substitute reagents from different kit lots. DO NOT mix up or substitute reagents from different manufacturers.

Components and storage

The kit contains sufficient reagents for 100 tests.

ID	Components	Size (100 tests)	Format	Storage (Unopened)	Storage (Opened)
IL2-H51P3	Human IL-2 / IL-4 / IL-6 / IL-10 / TNF-α / IFN-γ Protein	Refer to COA ×2	Powder	2-8 ℃	-70 ℃
FCMh021-C02A	Human IL-2 Capture Beads	100 tests	Beads suspension	2-8 ℃, avoid light	2-8 ℃, avoid light
FCMh021-C02B	Human IL-4 Capture Beads	100 tests	Beads suspension	2-8 ℃, avoid light	2-8 ℃, avoid light
FCMh021-C02C	Human IL-6 Capture Beads	100 tests	Beads suspension	2-8 ℃, avoid light	2-8 ℃, avoid light
FCMh021-C02D	Human IL-10 Capture Beads	100 tests	Beads suspension	2-8 ℃, avoid light	2-8 ℃, avoid light
FCMh021-C02E	Human TNF-α Capture Beads	100 tests	Beads suspension	2-8 ℃, avoid light	2-8 ℃, avoid light
FCMh021-C02F	Human IFN-γ Capture Beads	100 tests	Beads suspension	2-8 ℃, avoid light	2-8 ℃, avoid light
FCMh021-C03	Assay Buffer-03	60 mL	Liquid	2-8 ℃	2-8 ℃
FCMh021-C04	10 × Wash Buffer	10 mL	Liquid	2-8 ℃	2-8 ℃
FCMh021-C05	Detection Antibody	0.25 mL	Liquid	2-8 ℃, avoid light	2-8 ℃, avoid light
FCMh021-C06	96-well V-bottom Plate	1 plate	Solid	2-8 ℃	2-8 ℃
FCMh021-C07	96-well Sealing Film	2 pieces	Solid	2-8 ℃	2-8 ℃
FCMh021-C08	APC Positive Control	0.5 mL	Beads suspension	2-8 ℃, avoid light	2-8 ℃, avoid light
FCMh021-C09	PE Positive Control	0.5 mL	Beads suspension	2-8 ℃, avoid light	2-8 ℃, avoid light

Note：

- Store the unopened kit at 2–8 °C.
- Do not use the kit after the expiration date.
- Once reconstituted, the calibrator (IL2-H51P3) may undergo **no more than 2 freeze-thaw cycles**.

IMPORTANT: Bring all reagents to room temperature before use. If crystals have formed in buffer solution, place the buffer solution in an 37°C incubator until the crystals have completely dissolved and bring the solution back to room temperature before use.

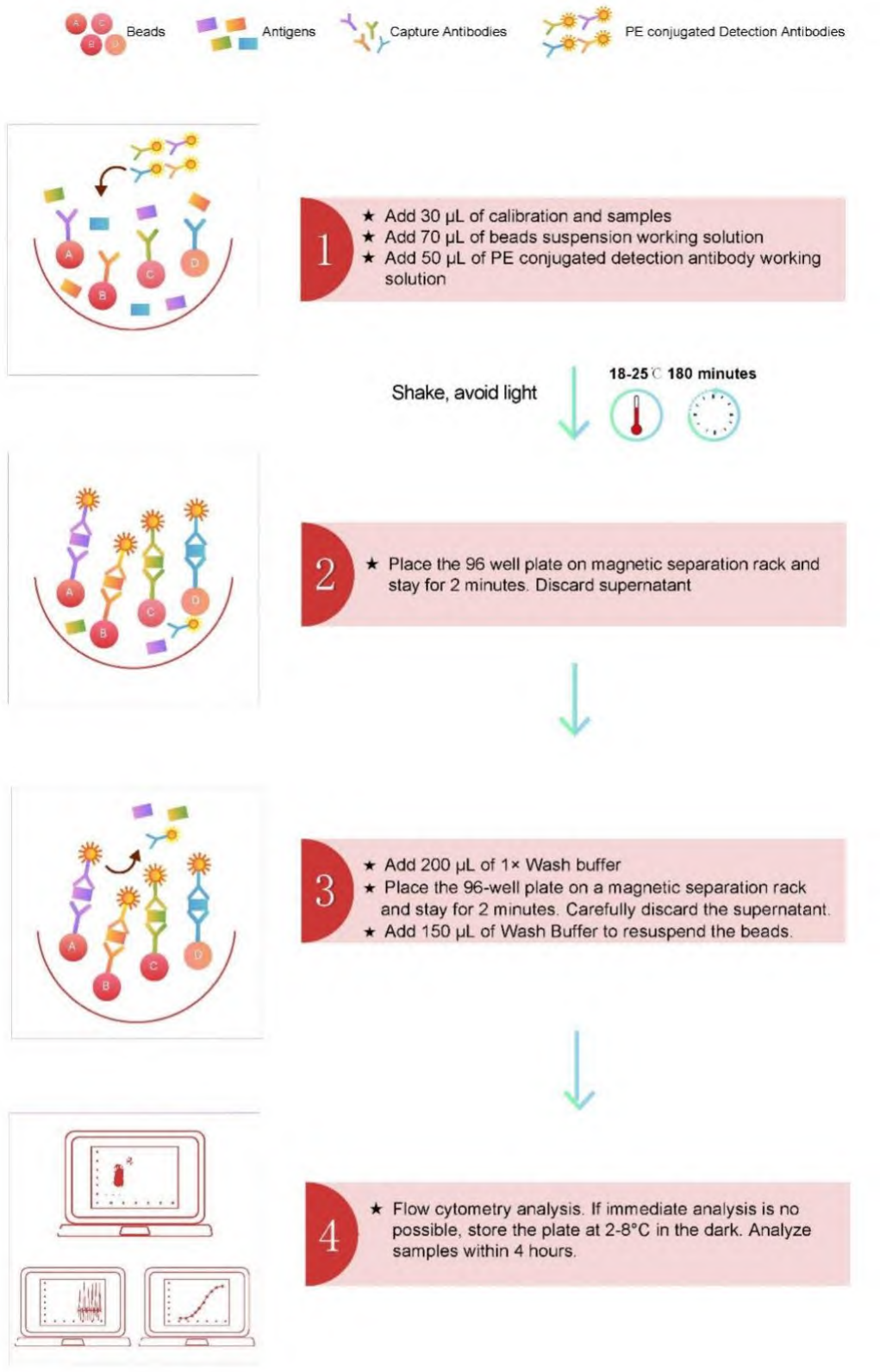
Required materials not supplied.

Instrument	• Flow cytometer equipped with two lasers: Excitation at 488 nm or 561 nm, emission around 575 nm; Excitation around 633 nm, emission around 670 nm • Vortex mixer • 96-well magnetic separation rack (Magnetic Capture Plate, catalog# FCM-C03M) • Horizontal orbital shaker for 96-well plate
Reagents	Deionized, ultrapure or distilled water
Consumables	50 mL and 500 mL graduated cylinders
	Pipettes, pipette tips and loading slot
	Tubes to prepare standard dilutions.

Precaution

All chemicals should be considered as potentially hazardous. It is recommended that this kit is handled only by those persons who have been trained in laboratory techniques and it is used in accordance with the principles of good laboratory practice. Suitable protective clothing such as laboratory overalls, safety glasses and gloves is needed. Attention should be paid to avoid contact with skin or eyes. In the case of contact with skin or eyes, wash immediately with plenty of water. All blood components and biological materials should be handled and disposed properly, in accordance with local and national guidelines.

Workflow


Beads
Antigens
Capture Antibodies
PE conjugated Detection Antibodies
1
★ Add 30 μL of calibration and samples
★ Add 70 μL of beads suspension working solution
★ Add 50 μL of PE conjugated detection antibody working solution
Shake, avoid light
18-25℃ 180 minutes
2
★ Place the 96 well plate on magnetic separation rack and stay for 2 minutes. Discard supernatant
3
★ Add 200 μL of 1× Wash buffer
★ Place the 96-well plate on a magnetic separation rack and stay for 2 minutes. Carefully discard the supernatant.
★ Add 150 μL of Wash Buffer to resuspend the beads.
4
★ Flow cytometry analysis. If immediate analysis is no possible, store the plate at 2-8°C in the dark. Analyze samples within 4 hours.

Preparation of samples

The kit is validated for use with **cell culture supernatant, serum, plasma**. If necessary, dilute samples with **Assay Buffer-03** to ensure values fall within the standard curve range. It recommended a 2-fold dilution of the samples to ensure optimal accuracy.

- **Cell culture supernatant**
  Freshly collected or -80 ℃ stored medium should be centrifuged at 4000 g for 10 minutes at 4 ℃, aspirate the supernatant for the next assay.
- **Serum collection and storage**
  Fresh blood samples were obtained from veins, keep at room temperature for more than 30 minutes. After coagulation, centrifuge at 2, 000 g for 10 minutes at 4 ℃ (Excessive centrifugation might lead to hemolysis). Aspirate the serum layer and avoid the contamination of blood cells. Serum layer was centrifuged at 16, 000 g for 10 minutes at 4 ℃. Discard the precipitates and the supernatant was the serum freshly prepared. Use the serum immediately or keep at -80 ℃ for long time storage.
- **Plasma collection and storage**
  Fresh blood samples were obtained from venous, using an anticoagulant such as EDTA, heparin, or sodium citrate. Centrifuge at 2, 000 g for 10 minutes at 4 ℃. Carefully aspirate the plasma layer, and avoid the contamination of blood cells. Centrifuge the plasma layer at 16, 000 g for 10 minutes at 4 ℃. Discard the precipitates, and keep supernatant as the freshly prepared plasma. Use the plasma immediately or store at -80 ℃.

Note: Frozen serum, plasma or medium should be mixed thoroughly after thawing, and remove all visible debris by centrifuge. Thawed samples should be used immediately and avoid repeated freeze-thaw cycles. Hemolyzed, icteric and lipemic samples are not validated for use in this assay.

Preparation of working solution and calibrator

- **Capture beads working solution**

  Vortex the bead suspensions vigorously for 10–30 seconds to ensure uniform dispersion. Based on **Table 1** calculate the required volume of each bead set and dilute with **Assay Buffer-03** to prepare the Capture Beads Working Solution. Add 70 µL of the Capture beads Working Solution to each well.

Table 1 Preparation of capture beads working solution

	1 Well	N Wells
Antibody-coupled APC-beads	1 µL for each Antibody-coupled APC-beads, total 6µL	N*1 µL for each Antibody-coupled APC-beads
Assay Buffer-03	64 µL	N*64 µL

Note: N is the number of wells.

- **Detection Antibody working solution**

  Prepare fresh immediately before use. Based on **Table 2,** calculate the required volume of each

detection antibody and dilute with **Assay Buffer-03** to prepare the Detection Antibody Working Solution. Add 50 µL of Detection Antibody Working Solution to each well.

Table 2 Preparation of detection antibody

	1 Well	N Wells
Detection Antibody	2.5 µL for Detection Antibody	N * 2.5 µL for Detection Antibody
Assay Buffer-03	47.5 µL	N * 47.5 µL

Note: N is the number of wells.

- **Wash Buffer working solution（1× Wash Buffer）**

  Add 10 mL of 10× Wash Buffer (**ID# FCMh021-C04**) to 90 mL of deionized water and mix thoroughly.

- **Reconstitution of lyophilized calibrator**

  Add 500 µL of deionized water to achieve a concentration of **1×10^7 pg/mL (Stock#1).** Incubate for 15 minutes at room temperature with occasional gentle mixing. ***Note: The standard concentrations of analytes in this kit, as reconstituted, are set at varying levels and may vary between production lots. For the exact values applicable to your kit, please download the lot-specific Certificate of Analysis (COA) from our official website.***

- **Prepare the calibrator serial dilutions.**

  Prepare a series of 1.5 mL microcentrifuge (EP) tubes. Perform serial dilutions of the standard with **Assay Buffer-03**, which also serves as the zero-concentration point (blank) of the standard curve. Prepare the dilutions fresh before use. Refer to the table below for the dilution scheme. **Vortex thoroughly after each dilution step.**

Table 3 Preparation of Calibrator

ID	Dilution Factor	Assay Buffer-03 (µL) add in	Calibrators add in (µL)	Concentration (pg/mL)
Stock#1				10,000,000
Stock#2	10	900	100 µL Stock#1	1,000,000
Stock#3	10	900	100 µL Stock#2	100,000
Stock#4	10	900	100 µL Stock#3	10,000
Cal 7	2	500	500 µL Stock#4	5000
Cal 6	2.5	600	400 µL Cal 7	2000
Cal 5	2.5	600	400 µL Cal 6	800
Cal 4	2.5	600	400 µL Cal 5	320
Cal 3	2.5	600	400 µL Cal 4	128.0
Cal 2	2.5	600	400 µL Cal 3	51.2
Cal 1	2.5	600	400 µL Cal 2	20.5
Cal 0		600	-	0

Assay Procedure

1. Add **30 µL** of calibrator (Cal 0–Cal 7) or test sample to each well of a 96-well V-bottom plate.

2. Add **70 µL** of capture beads working solution to each well.

3. Add **50 µL** of detection antibody working solution to each well. Seal the plate and incubate for **3 hours** at room temperature (18–25 °C) under continuous shaking at 600 rpm.

4. Place the plate on a magnetic separation rack for 2 min. Carefully aspirate the supernatant without disturbing the bead pellet.
(Alternative centrifugation method: seal the plate, centrifuge at 1000×g for 5 min, and aspirate the supernatant.)

5. Remove the plate from the rack, add 200 µL of 1× Wash Buffer to each well, and resuspend by pipetting up and down, then repeat step 4.

6. Resuspend the beads in 150 µL of 1× Wash Buffer and acquire on a flow cytometer.
Note: *Analyze samples within 4 hours after the final wash. If acquisition is delayed, store the plate at 2–8 °C, protected from light. Ensure the beads are fully resuspended to prevent aggregation during acquisition.*

7. Flow cytometer settings

   A dual-laser flow cytometer is suitable for this assay; validated instruments are listed in Table 4.
   Laser 1: excitation 488 nm or 561 nm; emission ~575 nm.
   Laser 2: excitation ~633 nm; emission 670 nm.

Table 4 Partial List of Compatible Flow Cytometers

Manufactures	Instrument	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 easyCyte3L	RED-R	YEL-B

8. Channel setup
   - Create a dot plot with FSC-A (X-axis) and SSC-A (Y-axis), and set both parameters to linear mode.
   - Create a histogram with APC on the X-axis. Vortex the APC positive control for 30 seconds and analyze at a low flow rate. Adjust FSC/SSC settings to clearly visualize the bead population, then set PMT voltages to ensure the APC signal falls within the detection range and preferably in the higher intensity region (Figure 2).

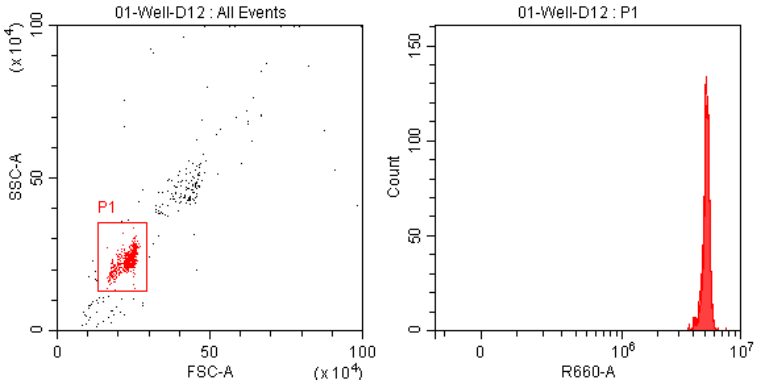


Figure 2 Optimization of PMT Voltages for FSC/SSC, APC Signals

- Create a histogram with PE on the X-axis. Vortex the PE positive control for 30 seconds and analyze at a low flow rate. Adjust FSC/SSC settings to clearly visualize the bead population, then set PMT voltages to ensure the PE signal falls within the detection range and preferably in the higher intensity region (Figure 3).

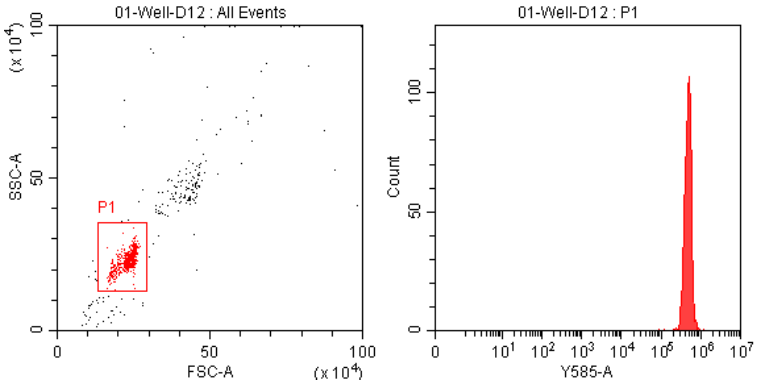


Figure 3 Optimization of PMT Voltages for FSC/SSC, PE Signals

9. Date acquisition

Note: *Ensure the flow cytometer is properly calibrated according to the instrument user guide and method configuration described above.*

- Set up the experiment in a 96-well plate format. Note: If a 96-well plate loader is unavailable, transfer the samples to 5 mL tubes, replenish with 100 µL 1× Wash Buffer, and read one by one.
- Select a medium flow rate for the samples, acquire 500 events or beads per target in the P1 gate, using this number as the stop criteria.
- Create a dot plot from P1 gate with APC on the X-axis and PE on the Y-axis to visualize different targets based on APC MFI intensity and PE signal.
- Load the plate and start acquisition. Identify each target based on the APC intensity and record the median fluorescence intensity (MFI) of PE channel (Figure 4).

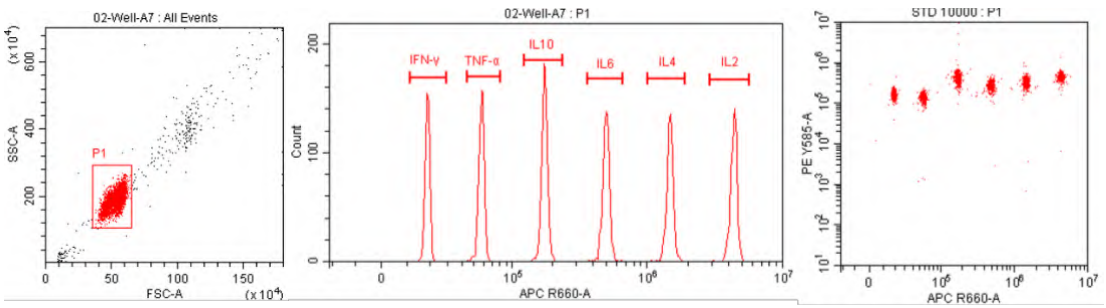


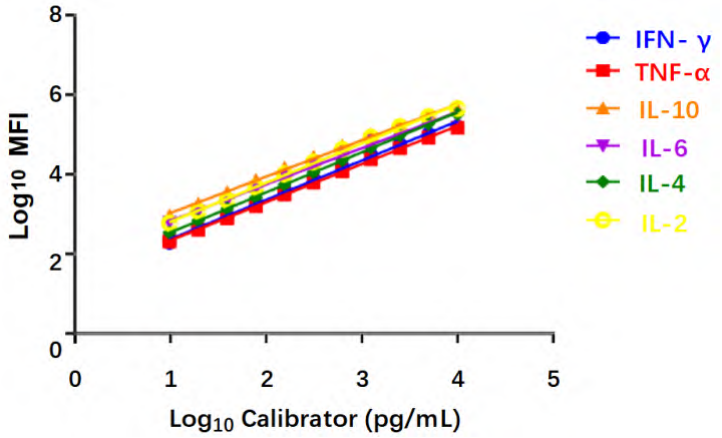
Figure 4 Standards of Analyte-specific Populations

Calculation of results

1. Export the PE median fluorescence intensity (MFI) values of all calibrators and samples.
2. Subtract the MFI of the 0 pg/mL calibrator from all calibrators and samples.
3. In GraphPad, plot the background-subtracted, Log10-transformed PE MFI values against the Log10-transformed calibrator concentrations, and fit **a log-log linear standard curve**. For reliable curve fitting, an R^2 value greater than 0.99 is recommended.
4. Calculate the concentrations of unknown samples from the standard curve for each analyte.
5. If the concentration of a test sample exceeds the upper limit of quantification (ULOQ) of the standard curve, dilute the sample with Dilution Buffer and repeat the assay.

Typical data

Note: For each experiment, a standard 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 standard curve. A double logarithmic fitting curve was plotted for each cytokine(IL-2, IL-4, IL-6, IL-10, TNF-alpha, IFN-gamma). The present curve of each cytokine was ranging from 20.5 pg/mL to 5000 pg/mL respectively.



LOD

The limit of detection (LOD) for each cytokine was calculated from 30 replicate blank wells (0 pg/mL). The mean MFI of the blanks plus 2 standard deviations (mean + 2×SD) was back-calculated to concentration using the log-log linear regression standard curve (20.5–5000 pg/mL).

Analyte	LOD (pg/mL)
IL-2	2.00
IL-4	2.62
IL-6	3.04
IL-10	0.28
TNF-α	2.47
IFN-γ	0.68

Biological sample values

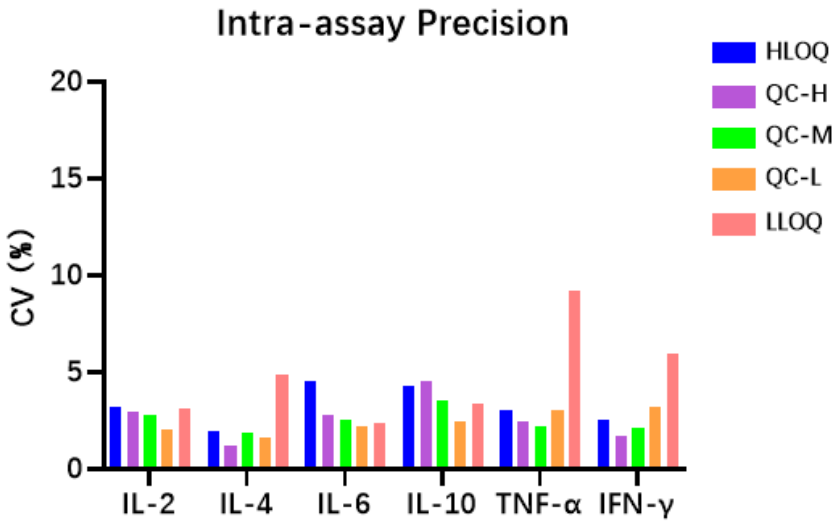
Human peripheral blood mononuclear cells (PBMCs) were cultured in RPMI 1640 supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 100 U/mL penicillin, and 100 µg/mL streptomycin sulfate. Cells were unstimulated or stimulated with 10 ng/mL of PMA and 500 ng/mL of Ionomycin Calcium Salt for 5 days. Cell culture supernatants were collected and analyzed using a 6-plex flow cytometry assay.

Cytokine	Condition	Day 1 Mean (pg/mL)	Day 3 Mean (pg/mL)	Day 5 Mean (pg/mL)
IL-2	**Unstimulated**	n.d.	31.33	56.43
	Stimulated	6,012.0	17,179.3	18,778.9
IL-4	**Unstimulated**	n.d.	n.d.	n.d.
	Stimulated	20.02	33.60	41.74
IL-6	**Unstimulated**	< 20.5	n.d.	< 20.5
	Stimulated	143.1	1,448.2	3,772.6
IL-10	**Unstimulated**	< 20.5	< 20.5	< 20.5
	Stimulated	n.d.	50.02	83.24
TNF-α	**Unstimulated**	26.99	< 20.5	< 20.5
	Stimulated	168.9	1,719.2	1,976.7
IFN-γ	**Unstimulated**	n.d.	< 20.5	< 20.5
	Stimulated	5,715.1	13,521.7	13,539.3

n.d.- Non-detectable

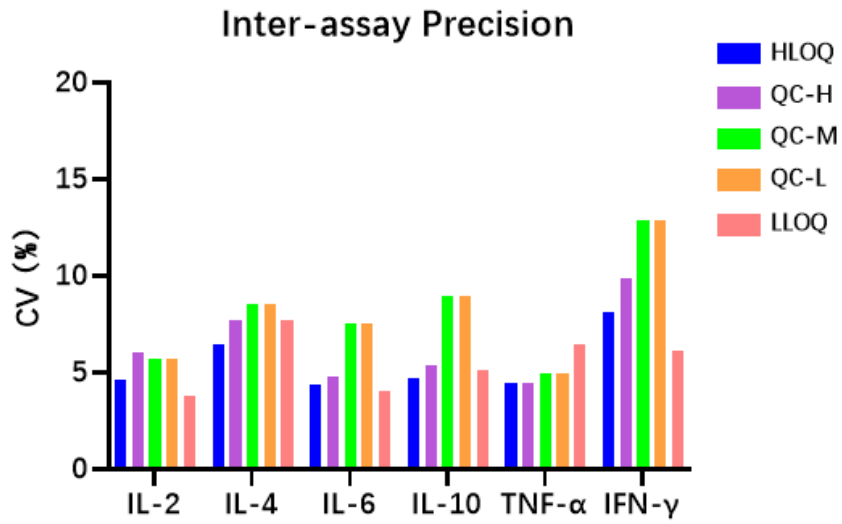
Intra-assay precision

Five concentration levels of target protein samples were each tested with 12 replicates in a single assay. Acceptable criteria: CV < 15%.



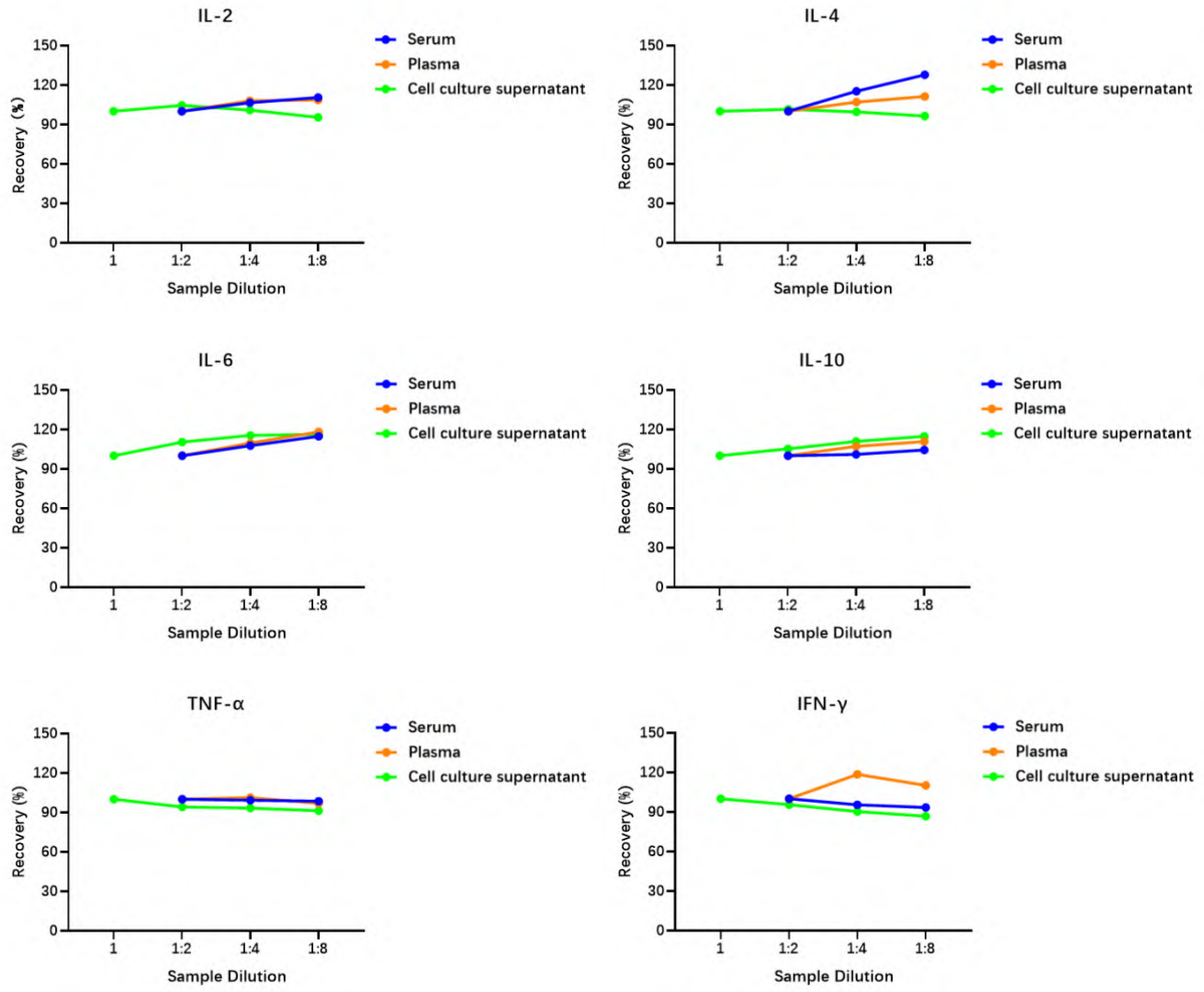
Inter-assay precision

The five concentration levels were tested in 3 independent experiments performed at different days .Acceptable criteria: CV < 20%.



Linearity

To assess the linearity of the assay, samples containing and/or spiked with high concentrations of human IL-2, IL-4, IL-6, IL-10, TNF-α and IFN-γ in various matrices were diluted with the assay buffer to produce samples with values within the dynamic range of the assay.



Recovery

Serum and plasma samples were diluted 2-fold with Assay Buffer; cell culture supernatant was tested undiluted. Target proteins were spiked at three concentration levels (3750.00 pg/mL, 312.50 pg/mL, 50.00 pg/mL). Recovery (%) was calculated as the ratio of the measured value to the theoretical value.

Cytokine	Serum Mean Recovery (%)	Plasma Mean Recovery (%)	Cell Culture Supernatant Mean Recovery (%)
IL-2	69-78	80-92	78-92
IL-4	59-68	73-82	85-93

IL-6	71-81	68-80	88-96
IL-10	86-88	80-86	94-99
TNF-α	94-100	95-103	103-109
IFN-γ	72-80	81-98	91-102

Specificity

Cross-reactivity was evaluated with recombinant proteins at a concentration of 1×10^5 pg/mL. All other analytes showed minimal or no cross-reactivity.

IL-1β	IL-2	IL-3	IL-4	IL-5	IL-6	IL-8
IL-10	IL-12	IL-13	IL-17A	IL-23	TNF-α	IFN-γ
GM-CSF	Fas	Fas L	Granzyme A	Granzyme B		

Trouble shooting

Concerns	Possible causes	Suggestions
The bead pellet is not visible or becomes smaller after repeated washing steps.	The beads are loosely captured by the magnet and may be lost during aspiration.	Aspirate the supernatant slowly and carefully. Leave the plate on the magnet longer until the beads are visibly concentrated.
Bead counts vary between replicate wells.	Aspiration takes too long, so the beads settle to the bottom of the tube or well before the suspension is dispensed.	Aspirate and dispense the bead suspension promptly. Vortex vigorously before first use, and vortex briefly between steps.
A large amount of debris is observed in the FSC-SSC scatter plot during acquisition.	The FSC/SSC threshold is set too low.	Raise the FSC and SSC threshold values.
A large number of bead doublets are observed in the FSC-H versus FSC-A plot.	The beads aggregate due to prolonged standing or inadequate resuspension.	Resuspend the beads by pipetting up and down vigorously, then reload onto the flow cytometer.
Fewer than 6 bead populations are resolved in the APC-count histogram.	The PMT gain or voltage of the APC channel is too high.	Lower the APC-channel PMT gain or voltage until 6 distinct peaks are observed.
Fewer than 6 bead populations are resolved in the APC-PE scatter plot, although all 6 peaks are resolved in the APC-count histogram.	The PMT gain or voltage of the PE channel is too high.	Lower the PE-channel PMT gain or voltage until 6 bead populations are resolved in the APC-PE scatter plot.
The PE fluorescence intensity of a low-concentration calibrator is higher than that of a high-concentration calibrator.	Insufficient needle washing between samples causes sample carryover.	Run at least one wash cycle between samples on the flow cytometer. Follow the recommended plate layout and acquire the plate by columns to reduce cross-contamination between samples.