Mouse TNF-α Detection Kit
(Flow Cytometry Bead Assay)

【Catalog】FCSm-027

【Size】100tests

Please read this manual carefully before performing the experiment.

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

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【Intended Use】

Mouse TNF-α Detection Kit (Flow Cytometry Bead Assay) gives quantitative results of TNF-α in biological samples (Serum, Plasma). The performance of this detection kit has been rigorously validated, including Linearity, LOD, Recovery and Precision. This kit can be multiplexed with other bead-based cytokine detection kits for simultaneous quantification of multiple cytokines in one sample.

【Principle】

Flow cytometric bead-based cytokine detection is a multiplex parallel immunoassay based on the sandwich antibody principle (Figure 1). Different bead populations are distinguished by particle size and internal APC fluorescence intensity (Figure 2). Capture beads and biotinylated detection antibodies targeting specific analytes are co-incubated with samples to bind the corresponding target analytes. Subsequently, streptavidin-phycoerythrin (SA-PE) is added to specifically bind to the biotin moieties on the detection antibodies. Ultimately, a sandwich complex of capture bead–analyte–detection antibody–biotin–SA-PE is formed on the bead surface. The Median Fluorescence Intensity (MFI) of PE is positively correlated with the concentration of target analytes in the sample.

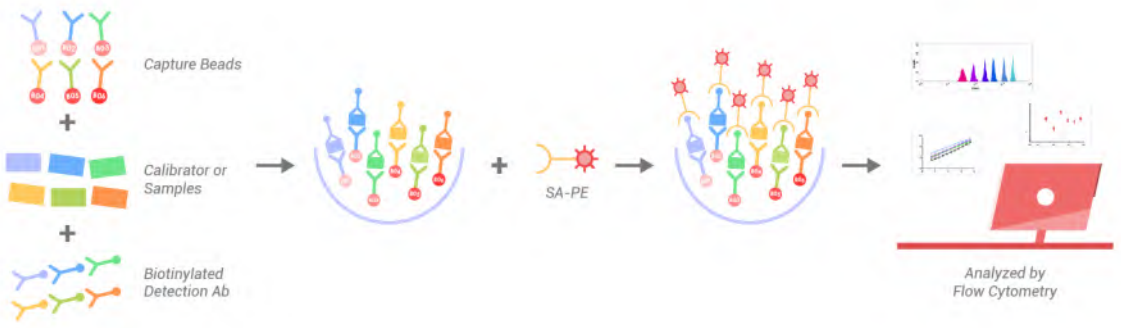

Figure 1. Principle of Multiplex Bead Assay

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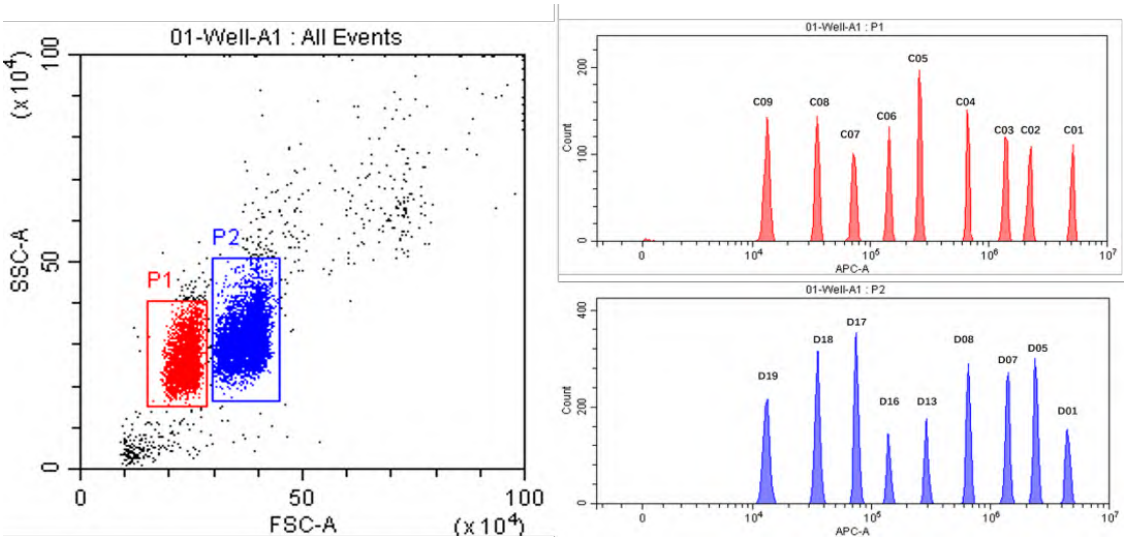

Figure 2. Bead populations: Bead populations are distinguished by their distinct sizes, appearing as separate clusters (P1 and P2) on the FSC-A/SSC-A dot plot (left), and by their specific APC fluorescence intensities, which are shown as distinct peaks on the APC-A histogram (right). The relative position of each target in the histogram can be identified by the coding suffix included in the corresponding bead product name.

【Components】

Cat. No.	Components	Size	Format	Storage (unopen)	Storage (open)
FMBc036-02	Anti-Mouse TNF-α Antibody-coupled APC-beads-D13	50 tests×2	Bead suspension	2-8 °C, avoid light	2-8 °C, avoid light
FABr019-B	Biotinylated Monoclonal Anti-Mouse TNF-a Antibody, Rabbit IgG (006)	0.25mL×2	Liquid	2-8 °C, avoid light	2-8 °C, avoid light
IL2-M51P3	Mouse IL-2 / IL-4 / IL-6 / IL-10 / TNF-α / IFN-γ Protein	5 μg×2	Powder	2-8 °C	-70°C
STN-NP119	Streptavidin Protein-PE	100 μg	Powder	2-8 °C, avoid light	2-8 °C, avoid light
FCMB-03	Assay Buffer-01	50 mL×2	Liquid	2-8 °C	2-8 °C
FCMB-04	Wash Buffer-01	50 mL×2	Liquid	2-8 °C	2-8 °C
FCMP-01	96-Well V-Bottom Assay Plate	1 plate	Solid	2-8 °C/RT	2-8 °C/RT
FCMP-02	96-well Sealing film	2 pieces	Solid	2-8 °C/RT	2-8 °C/RT
FMBf001-01	PE Positive Control-01	0.5 mL	Bead suspension	2-8 °C, avoid light	2-8 °C, avoid light
FMBf001-02	APC Positive Control-01	0.5 mL	Bead suspension	2-8 °C, avoid light	2-8 °C, avoid light

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【Storage and Expiration】

1. Store unopened kit components at 2~8 ℃.
2. Check the expiration date on the package; do not use reagents past their expiration.
3. Once reconstituted, prepare small aliquots (≥30 µL per aliquot) and store at -70 °C to avoid repeated freeze-thaw cycles.

【Unsupplied Materials and Instruments】

Materials and Instruments	Specification
Single-channel pipettes	100µL~1mL, 20µL~ 200µL, 2µL ~20µL
multi-channel pipettes	30~300µL, 10µL ~50µL
pipette tips	1mL, 200µL, 20µL
Reagent reservoirs for multichannel pipette	/
Polypropylene microcentrifuge tubes	1.5mL, 15mL
Deionized or distilled ultrapure water	/
Horizontal orbital shaker	≥600prm
Vortex mixer	/
Flow cytometer	Equipped with two lasers: (1) Excitation at 488 nm or 561 nm, emission at 575 nm; (2) Excitation at 633 nm, emission at 670 nm

【Important Information】

1. For Research Use Only. Not for diagnostic or therapeutic applications.
2. Strictly follow the instructions to ensure optimal and consistent data output.
3. Protect bead suspensions and detection antibodies from light at all times to prevent photobleaching.
4. Do not mix or substitute reagents from different kit lots or manufacturers.
5. Bring all kit components to room temperature before use.
6. Prepare all buffers, reagents, calibrators, samples, and related materials immediately prior to use.
7. Use deionized or distilled water for all reagent preparations.
8. Ensure all reagent reservoirs are clean and dry.
9. Use disposable pipette tips to prevent microbial contamination or cross-contamination of reagents/specimens, which may invalidate test results.

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【Safety Precautions】

1. All chemicals should be regarded as potentially hazardous.
2. This kit should be handled only by trained laboratory personnel and used in compliance with Good Laboratory Practice (GLP) principles.
3. Wear appropriate personal protective equipment (PPE), including lab coats, safety goggles, and gloves.
4. Avoid skin or eye contact. In case of contact, rinse immediately with plenty of water and seek medical advice.
5. Handle and dispose of all blood components and biological materials in accordance with local/national regulations.

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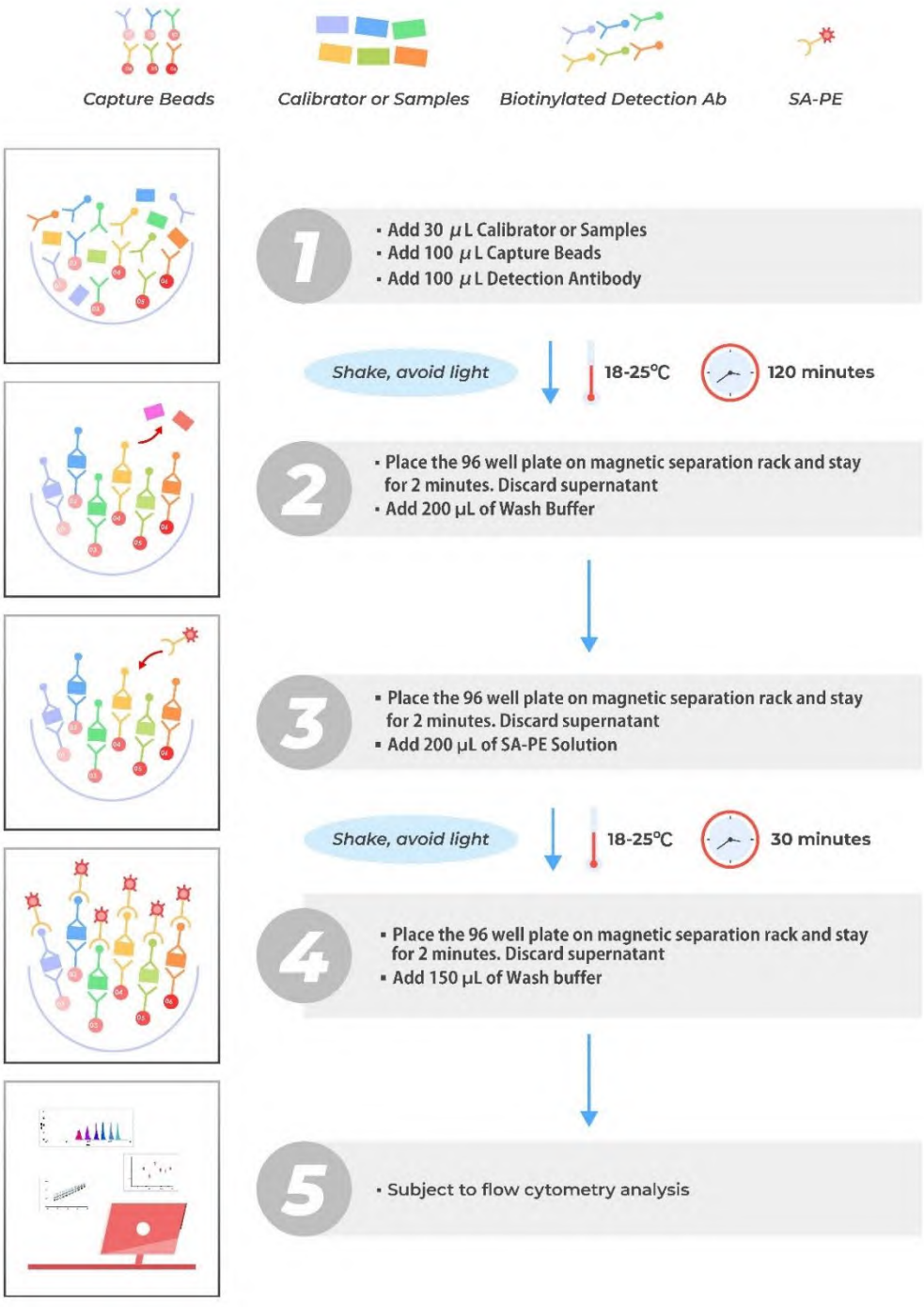
【Quick Guide】



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【Assay procedure】

1. Assay Preparation

1.1 Preparation of Reagents and Buffer

1.1.1 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-01 (**ID#FCMB-03, this buffer is supplied as 1×Assay Buffer and is ready for use without dilution**) to prepare the Capture Beads Working Solution. Add 100 µL of the Capture Beads Working Solution to each well.

Table 1. Preparation of Capture Beads

	1 Well	Y Wells
Antibody-coupled APC-beads	5 µL for each Antibody-coupled APC-beads	Y*5 µL for each Antibody-coupled APC-beads
Assay Buffer-01	(100-X*5) µL	Y*(100-X*5) µL

Note: *X is the number of targets (for example, for the detection of IL-2/IL-4/IL-6, X is 3). Y is the number of wells.*

1.1.2 Detection Antibody Working Solution

Based on Table 2, calculate the required volume of each detection antibody and dilute with Assay Buffer-01 to prepare the Detection Antibody Working Solution. Add 100 µL of Detection Antibody Working Solution to each well.

Table 2. Preparation of Detection Antibody

	1 Well	Y Wells
Biotin-labeled Antibody	5 µL for each Biotin-labeled Antibody	Y*5 µL for each Biotin-labeled Antibody
Assay Buffer-01	(100-X*5) µL	Y*(100-X*5) µL

Note: *X is the number of targets (for example, for the detection of IL-2/IL-4/IL-6, X is 3). Y is the number of wells.*

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1.1.3 SA-PE Solution

Prepare fresh immediately before use. Reconstitute the lyophilized Streptavidin–Phycoerythrin (SA-PE) powder with 200 µL of deionized water. Incubate at room temperature for 15 minutes to obtain a stock solution with an initial concentration of 500 µg/mL. Dilute the reconstituted SA-PE stock to a working concentration of 1.5 µg/mL with Assay Buffer-01, as shown in Table 3. Each well requires 200 µL of SA-PE Solution.

Table 3. Preparation of SA-PE Solution

	1 Well	**Y Wells**
Reconstructed Streptavidin Protein-PE (Conc. 500 µg/mL)	0.6 µL	Y*0.6 µL
Assay Buffer-01	200 µL	Y*200 µL

Note: *Y is the number of wells.*

1.2 Preparation of Calibrator

1.2.1 Reconstitution of Lyophilized Calibrator

1.2.1.1 Mouse IL-2 / IL-4 / IL-6 / IL-10 / TNF-α / IFN-γ Protein: 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: Once the calibrator is reconstituted, prepare small aliquots (≥30 µL per aliquot) and store the stock solution at -70°C immediately and avoid repeated freeze-thaw cycles.

1.2.2 Serial Dilution Steps

1.2.2.1 Combine 900 µL Assay Buffer, 100 µL Stock#1 to create **Stock#2** (1×10^6 pg/mL).

1.2.2.2 Combine 900 µL of Assay Buffer with 100 µL of Stock#2 to create **Stock#3** (1×10^5 pg/mL).

1.2.2.3 Combine 900 µL of Assay Buffer with 100 µL of Stock#3 to create **Cal 11** (1×10^4 pg/mL).

1.2.2.4 Perform two-fold serial dilutions from Cal 11, labeling samples Cal 10 through Cal 1 (refer to Table 4).

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Note: Vigorously mix each dilution to ensure homogeneity.

Table 4. Preparation of Calibrator

Calibrator ID	Serial Dilution	Assay Buffer Add in (µL)	Calibrator Add in (µL)	Final Concentration (pg/mL)
Cal 11				10,000
Cal 10	2	500	500 µL of Cal 11	5,000
Cal 9	2	500	500 µL of Cal 10	2,500
Cal 8	2	500	500 µL of Cal 9	1250
Cal 7	2	500	500 µL of Cal 8	625
Cal 6	2	500	500 µL of Cal 7	312.5
Cal 5	2	500	500µL of Cal 6	156.3
Cal 4	2	500	500 µL of Cal 5	78.1
Cal 3	2	500	500 µL of Cal 4	39.1
Cal 2	2	500	500 µL of Cal 3	19.5
Cal 1	2	500	500 µL of Cal 2	9.8
Cal 0	-	500	-	0

2. Assay Procedure

2.1 Sample Addition: Add serial dilutions of Calibrator (Cal 0 - Cal 11) or samples to 96-well V-bottom plate, 30 µL/well.

2.2 Capture Beads Addition: Add 100 µL of Capture Beads Working Solution to each well.

2.3 Detection Antibody Addition: Add 100 µL of Detection Antibody Working Solution to each well.

2.4 Primary Incubation: Seal the plate and incubate at room temperature for 120 minutes with continuous shaking at 600~800 rpm.

2.5 Magnetic Separation and Washing: After incubation, place the plate on the magnetic separation rack for 2 minutes. Carefully aspirate the supernatant without disturbing the bead pellet. Remove the plate from the magnet, add 200 µL of Wash Buffer-01 (**ID#FCMB-04, this buffer is supplied as 1×Wash Buffer and is ready for use without dilution**) to each well. Repeat the magnetic separation and aspiration steps.

(**Alternative Centrifugation Method**: Seal the plate and centrifuge at 1000×g for 5 minutes.

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Aspirate the supernatant, resuspend the beads in 200 µL of Wash Buffer-01. Repeat centrifugation and aspiration steps.)

2.6 **SA-PE Addition:** Add 200 µL of SA-PE Solution to each well.

2.7 **Secondary Incubation:** Seal the plate and incubate at room temperature for 30 minutes with continuous shaking at 600~800 rpm.

2.8 **Washing Step:** Repeat the washing step 2.5. Resuspend the bead in 150 µL of Wash Buffer-01.

2.9 **Flow Cytometry Analysis:** Proceed with flow cytometry analysis.

***Note**:*

1) Analyze samples within 4 hours after the final wash step.

2) If immediate analysis is not possible, store the plate at 2-8°C in the dark.

3) Ensure beads are fully resuspended to prevent clumping during flow cytometry analysis.

3. Flow Cytometer Setup

3.1 Flow cytometer equipped with two lasers are compatible with this assay:

Laser 1: Excitation at 488 nm or 561 nm, emission at approximately 575 nm;

Laser 2: Excitation at approximately 633 nm, emission at 670 nm.

Flow cytometers validated for use with this assay are listed in Table 5.

Table 5. Partial List of Compatible Flow Cytometers

Manufacturer	Verified instrument model	Classification Channel	Reporter Channel
BD Biosciences	BD FACSLyric™	APC	PE
BD Biosciences	BD FACSymphony™ A1	APC	PE
Beckman Coulter	Cytoflex S	R660-APC	Y585-PE

3.2 Set up PMT Voltages

3.2.1 Create a dot plot with FSC-A (X-axis) and SSC-A (Y-axis) , and set both parameters to linear mode.

3.2.2 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

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and preferably in the higher intensity region.

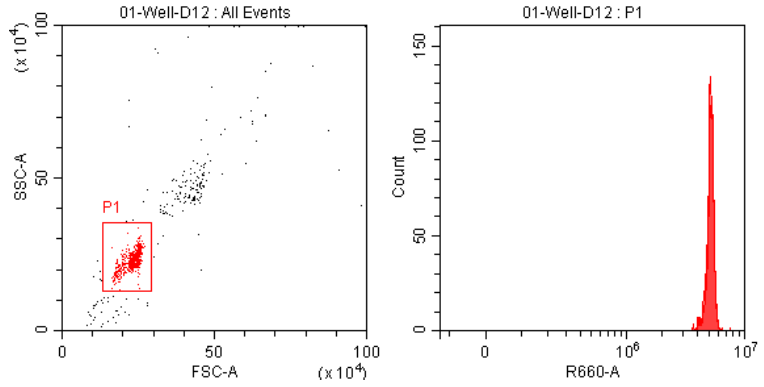

Figure 3. Optimization of PMT Voltages for FSC/SSC, APC Signals

3.2.3 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.

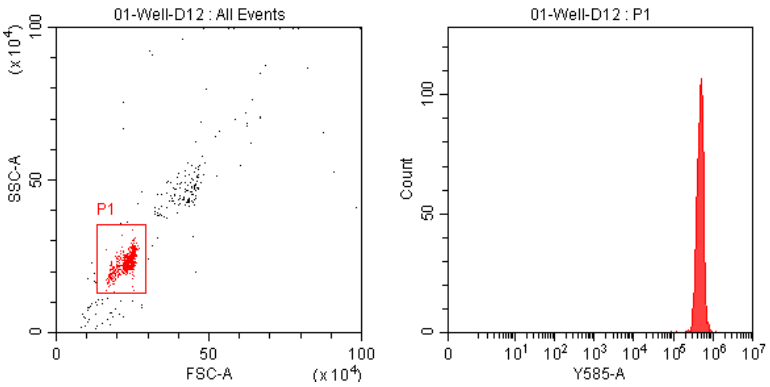

Figure 4. Optimization of PMT Voltages for FSC/SSC, PE Signals

4. Data Acquisition and Analysis

4.1 Data acquisition

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

4.1.2 Set up the experiment in a 96-well plate format.

Note: *If a 96-well plate loader is unavailable, transfer the samples to FACS tubes, replenish with*

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100 μL Wash Buffer, and read one by one.

4.1.3 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.

4.1.4 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.

4.1.5 Load the plate and start acquisition.

4.1.6 Identify each target based on the APC intensity and record the median fluorescence intensity (MFI) of PE channel.

4.2 Data analysis

4.2.1 Data Export: Export the PE median fluorescence intensity (MFI) values of all standards and samples.

4.2.2 Background Subtraction: Subtract the MFI value of the 0 pg/mL standard from all calibrators and samples.

4.2.3 Standard Curve Fitting: Generate a two-log-linear standard curve using GraphPad software by plotting Log10-transformed calibrator concentrations against Log10-transformed PE MFI values after background subtraction. For reliable curve fitting, an R^2 value greater than 0.99 is recommended.

4.2.4 Quantification: Calculate the concentrations of unknown samples using the standard curve for each analyte.

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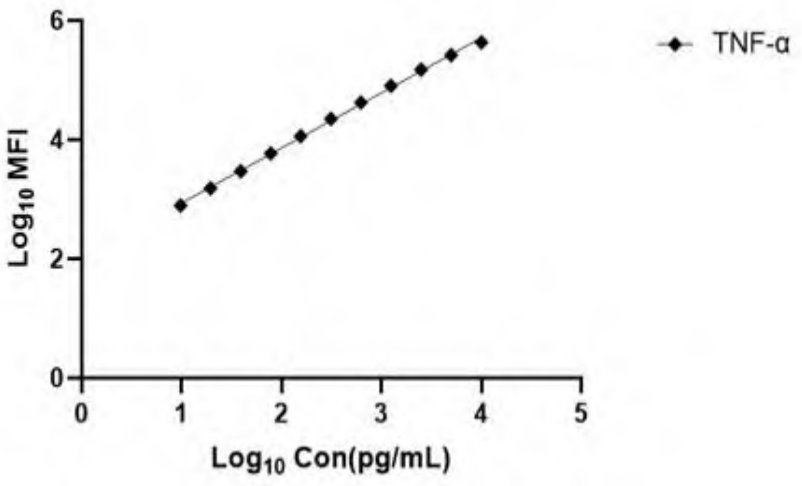
【Typical Data】



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【Performance】

1. Limit of Detection

The theoretical limit of detection is defined as the corresponding concentration at two standard deviations above the mean fluorescence of ⩾30 replicates of the negative control (0 pg/mL).

	Concentration(pg/ml)
LOD	0.45

2. Recovery

Recombinant Mouse TNF-α protein was spiked into serum（pool⩾10）, plasma（pool⩾10）at three concentrations (7500 pg/mL, 312.5 pg/mL, and 25 pg/mL). The measured values of the spiked samples were compared with the expected concentrations, as shown below.

Samples	Spike concentration（pg/mL）	Recovery
Serum	25	77%
	312.5	83%
	7500	84%
Plasma	25	85%
	312.5	88%
	7500	78%

3. Specificity

No cross-reactivity was observed between the Mouse TNF-α and the following recombinant cytokines.

IL-17A	IFN-γ	IL-6
IL-4	IL-10	IL-2
IL-5	IL-12p70	GM-CSF
IL-1β	MCP-1	

4. Intra-assay precision

3 samples with different concentration（7500 pg/mL, 312.5 pg/mL, 25 pg/mL）of target proteins were analyzed in one assay with 6 replicates for each sample. The intra-assay precision was calculated as below.

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Sample	%CV
Sample 1-25 pg/mL	2%
Sample 2-312.5 pg/mL	6%
Sample 3-7500 pg/mL	2%

5. Inter-assay precision

3 samples with different concentration（7500 pg/mL, 312.5 pg/mL, 25 pg/mL）of target proteins were analyzed in three independent assays with 3 replicates for each sample. The inter-assay precision was calculated as below.

Sample	%CV
Sample 1-25 pg/mL	11%
Sample 2-312.5 pg/mL	3%
Sample 3-7500 pg/mL	3%

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【Trouble Shooting】

Concerns	Possibilities	Suggestions
After magnetic separation, the magnetic beads precipitates are not visible or become less and less after multiple-step operation.	The pellets are very loosely attached to the well, and lost during aspiration.	Aspirate the supernatant slowly and carefully. Keep the plate at magnet for a long time with a visible accumulation of the beads.
Variation of beads count in duplicated wells.	Aspiration takes so long time that the beads settled to the bottom of the tube or wells.	Quickly aspirate and dispense the bead suspension. Vortex beads vigorously before first use, and vortex briefly in between operation.
Plenty of debris were observed in FSC-SSC scatter plot during data acquisition.	Improper setting of FSC and SSC threshold.	Increase threshold value of FSC and SSC.
Plenty of bead doublets are observed by plotting FSC height versus FCS area.	Beads aggregate due to long time sitting or insufficient resuspending.	Resuspend the beads by pipetting up and down vigorously, then re-load onto flow cytometer.
PE fluorescent intensity of low concentration calibrator is higher than that of high concentration calibrator.	Insufficient needle wash and clean between samples.	At least one washing cycle between samples in flow cytometer setting.
		Follow the Plate Layout suggested, and read the plate by columns to reduce cross-contamination on flow cytometer.

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