Buffer Set-01 (Flow Cytometry Bead Assay)

【Catalog】FCMB-05

【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】

The Multiplex Bead Assay is a versatile and efficient method for protein quantification, compatible with serum, plasma, and cell culture supernatants. Its key advantage lies in multiplexing, enabling simultaneous detection of multiple targets in a single assay, thereby maximizing data output while conserving samples. Compared with conventional ELISA, the Multiplex Bead Assay requires less sample volume and reduces assay time, hands-on effort, and overall cost by integrating multiple analyses into a single run. In contrast, single-plex ELISA is more time-consuming, labor-intensive, and resource-intensive.

A comprehensive portfolio of target-specific reagents further enables flexible panel customization to meet diverse research needs.

【Principle】

Flow cytometric bead-based cytokine detection is a multiplex immunoassay based on the sandwich antibody principle (Figure 1). Distinct bead populations, differentiated by size and internal APC fluorescence intensity (Figure 2), are each coated with a capture antibody specific to a target cytokine and incubated with samples to bind the corresponding analytes. A PE-labeled detection antibody is then added to form a fluorescent sandwich complex. During flow cytometric analysis, each bead population is identified by its size and internal APC fluorescence characteristics, while the reporter PE fluorescence intensity (median fluorescence intensity, MFI) is directly proportional to the cytokine concentration, enabling quantitative determination using a standard curve.

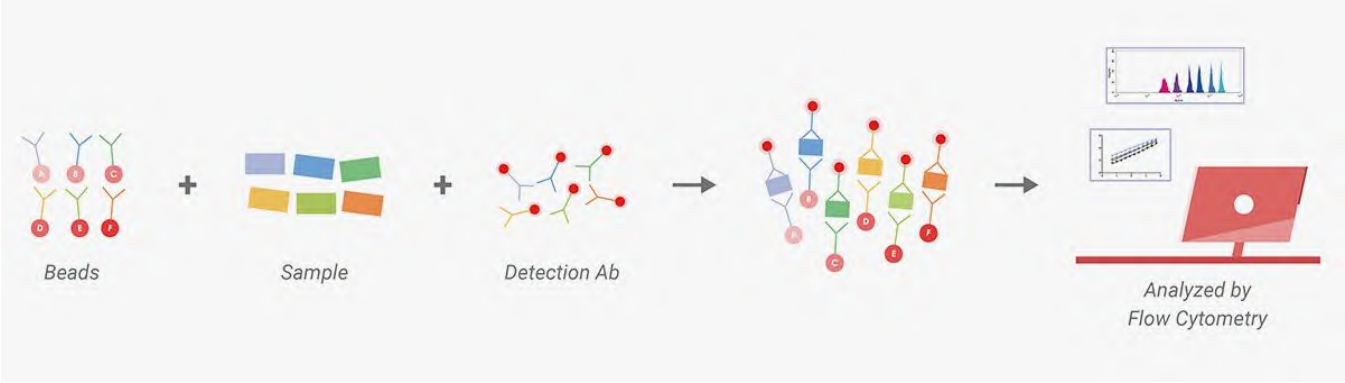

Figure 1. Principle of Multiplex Bead Assay

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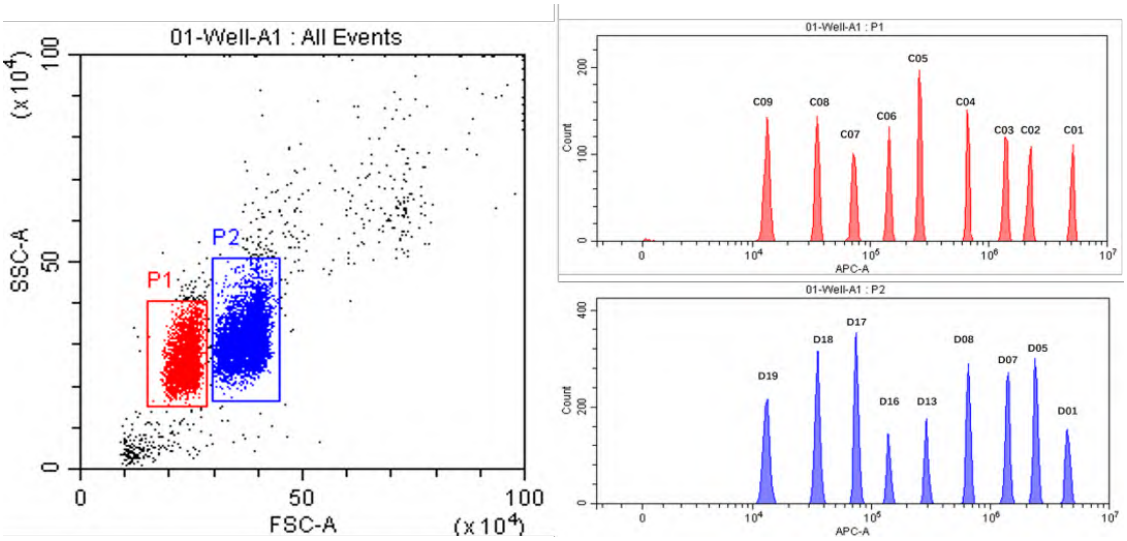

Figure 2. Bead populations: Bead populations are distinguished by their distinct sizes, appearing as separate clusters (P1 and P2) on the FSC-A/SSC-A dot plot (left), and by their specific APC fluorescence intensities, which are shown as distinct peaks on the APC-A histogram (right). The relative position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

【Components】

Please refer to the Certificate of Analysis (COA) for details.

【Unsupplied Materials and Instruments】

Materials and Instruments	Specification
Single-channel pipettes	100 µL~1 mL, 20 µL~ 200 µL, 2 µL ~20 µL
multi-channel pipettes	30 µL~300 µL, 10 µL ~50 µL
pipette tips	1 mL, 200 µL, 20 µL
Reagent reservoirs for multichannel pipette	/
Polypropylene microcentrifuge tubes	1.5 mL, 15 mL
Deionized or distilled ultrapure water	/
Horizontal orbital shaker	≥600 prm
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

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【Storage】

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.

【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.

【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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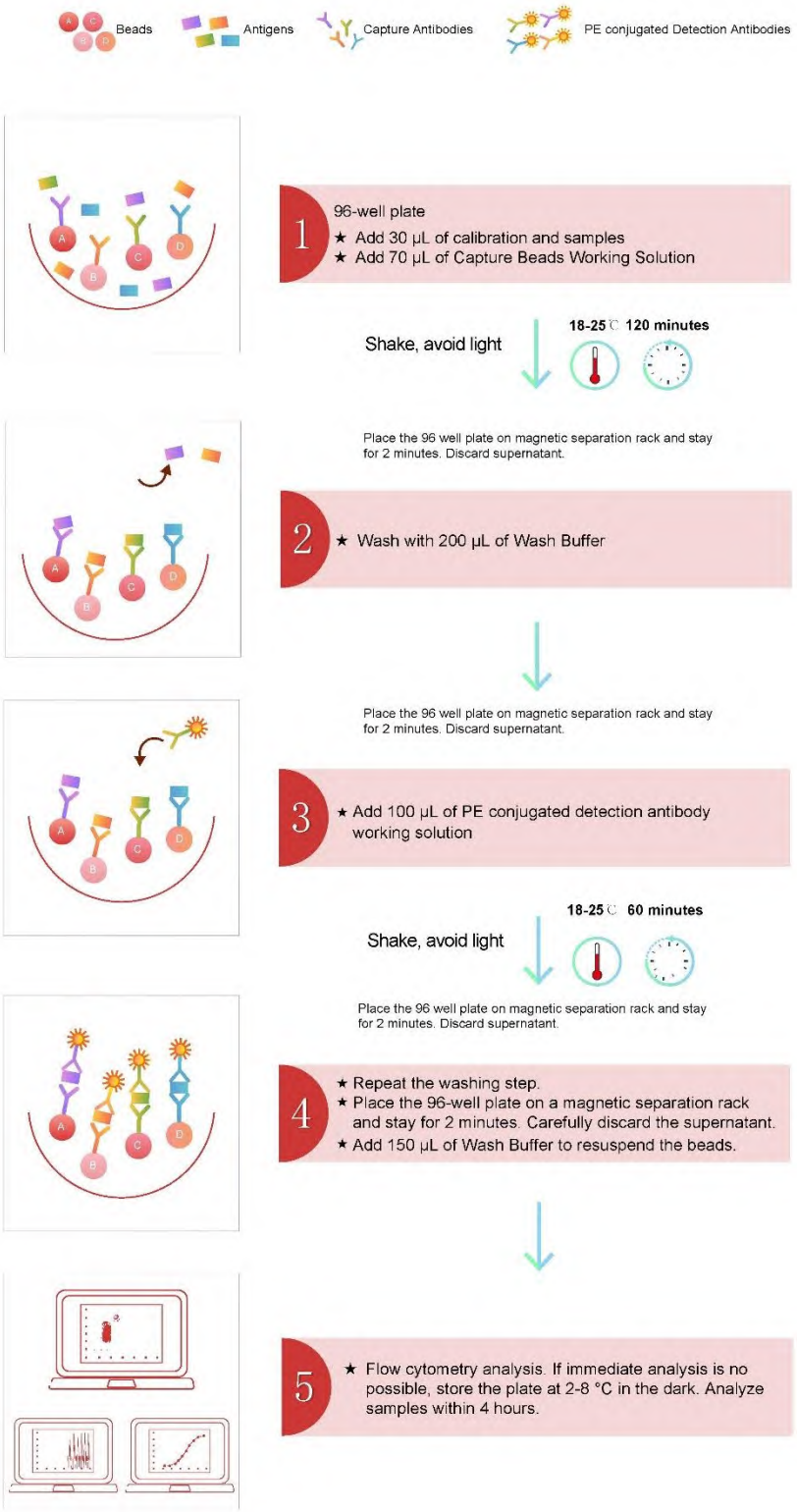
【Quick Guide】



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【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 70 µL of the Capture Beads Working Solution to each well.

Table 1. Preparation of Capture Beads

	1 Well	Y Wells
Antibody-coupled APC-beads	3.5 µL for each Antibody-coupled APC-beads	Y*3.5 µL for each Antibody-coupled APC-beads
Assay Buffer	(70-X*3.5) µL	Y*(70-X*3.5) 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

Prepare fresh immediately before use. 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
PE-labeled Antibody	5 µL for each PE-labeled Antibody	Y*5 µL for each PE-labeled Antibody
Assay Buffer	(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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1.2 Calibrator Preparation

1.2.1 Reconstitution of Lyophilized Calibrator

Reconstitute the lyophilized calibrator with the specified volume of deionized water according to the kit COA, and allow it to stand at room temperature for 15 minutes. Gently mix reagents to avoid bubbles and foam.

1.2.2 Serial Dilution Steps

1.2.2.1 Take 100 µL of each reconstituted calibrator (diluted to 1×10^7 pg/mL) and adjust the total volume to 1000 µL with Assay Buffer-01 to create **Stock#1** (1×10^6 pg/mL).

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

1.2.2.3 Combine 900 µL of Assay Buffer with 100 µL of Stock#2 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 3).

***Note**: Vigorously mix each dilution to ensure homogeneity.*

Table 3. Preparation of Calibrator

Calibrator ID	Serial Dilution	Assay Buffer Add in (µL)	Calibrator Add in (µL)	Final Concentration (pg/mL)
Stock#1				1,000,000
Stock#2	10	900	100 µL of Stock#1	100,000
Cal 11	10	900	100 µL of Stock#2	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

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1.3 Sample Preparation

The kit is validated for use with cell culture supernatant, serum, plasma. If necessary, dilute samples with Assay Buffer-01 to ensure values fall within the standard curve range. We recommend a 2-fold dilution of the samples to ensure optimal accuracy.

1.3.1 Cell Culture Supernatants

Freshly collected culture media or samples stored at −80 °C should be centrifuged at 4000 × g for 10 minutes at 4 °C. Carefully collect the supernatant for subsequent analysis.

1.3.2 Serum Collection and Storage

1.3.2.1 Collect fresh blood samples via venipuncture and allow to stand at room temperature for at least 30 minutes. After clotting, centrifuge at 2000 × g for 10 minutes at 4 °C (excessive centrifugal force may cause hemolysis). Carefully collect the serum layer, avoiding contamination with blood cells.

1.3.2.2 Centrifuge the collected serum at 16000 × g for 10 minutes at 4 °C. Discard the pellet; the supernatant is freshly prepared serum.

1.3.2.3 Serum should be used immediately or stored at −80 °C for long-term storage.

1.3.3 Plasma Collection and Storage

1.3.3.1 Collect fresh blood samples via venipuncture into tubes containing anticoagulants (e.g., sodium citrate, EDTA, or heparin). Centrifuge at 2000 × g for 10 minutes at 4 °C. Carefully collect the plasma layer, avoiding contamination with blood cells.

1.3.3.2 Centrifuge the plasma at 16000 × g for 10 minutes at 4 °C. Discard the pellet; the supernatant is freshly prepared plasma. Plasma should be used immediately or stored at −80 °C.

Note 1: *Frozen serum, plasma, or culture media should be fully thawed, thoroughly mixed, and centrifuged to remove all visible particulates before use. Thawed samples should be used immediately and should not undergo repeated freeze–thaw cycles.*

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

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2. Assay Procedure

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

2.2 **Capture Beads Addition:** Add 70 µL of Capture Beads working solution to each well.

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

2.4 **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-03, this buffer is supplied as 1×Assay 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. Aspirate the supernatant, resuspend the beads in 200 µL of Wash Buffer-01. Repeat centrifugation and aspiration steps.)

2.5 **Detection Antibody Addition:** Add 100 µL of Detection Antibody to each well.

2.6 **Secondary Incubation:** Seal the plate and incubate at room temperature for 60 minutes with continuous shaking at 600 rpm.

2.7 **Washing Step:** Repeat the washing step 2.4. Resuspend the bead in 150 µL of Wash Buffer-01.

2.8 **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.

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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 4.

Table 4. 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 and preferably in the higher intensity region (Figure 3).

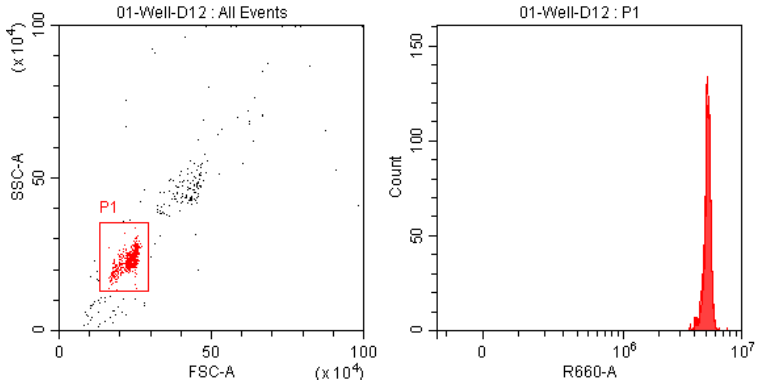

Figure 3. Optimization of PMT Voltages for FSC/SSC, APC Signals

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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).

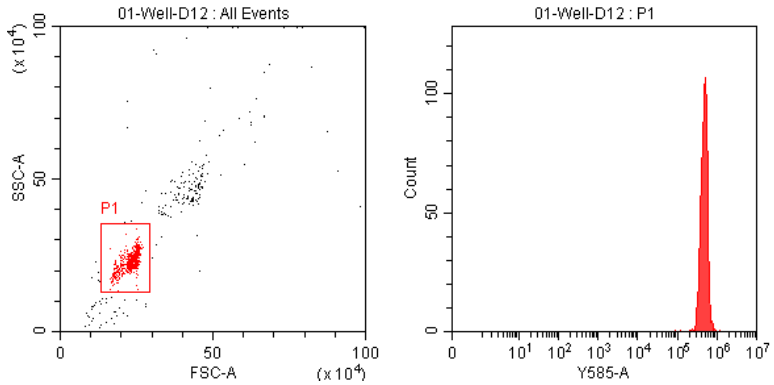

Figure 4. Optimization of PMT Voltages for FSC/SSC, PE Signals

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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 5mL tubes, replenish with 100 µL Wash Buffer-01, 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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【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 slowly and carefully. Ensure beads accumulate visibly on the magnet for optimal separation.
Variation of beads count in duplicated wells.	Aspiration takes so long time that the beads settled to the bottom of the tube or wells.	Vortex beads before use and briefly between operations to ensure uniform resuspension.
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.
Less than planned bead populations in APC-count histogram.	The PMT gain or voltage value of APC fluorescent channel is too high.	Adjust PMT gain or voltage of APC fluorescent channel, ensuring all intact peaks observed.
Less than planned bead populations in APC-PE scatter plot, though all peaks obtained	The PMT gain or voltage value of PE fluorescent channel is too high.	Adjust PMT gain or voltage of PE fluorescent channel, ensuring all bead populations in APC-PE scatter plot.

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in APC-count histogram.		
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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