Human Th1/Th2 Cytokine Panel (Flow Cytometry Multiplex Bead Assay)
Human Th1/Th2/Th17 Cytokine Panel (Flow Cytometry Multiplex Bead Assay)
For Individual Reagents

Please read this manual carefully before performing the experiment.

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

Catalog

【Intended Use】

Human Th1/Th2 Cytokine Panel (Flow Cytometry Multiplex Beads Assay) gives quantitative results of Interleukin-2 (IL-2), Interleukin-4 (IL-4), Interleukin-6 (IL-6), Interleukin-10 (IL-10), Tumor Necrosis Factor α type (TNF-α) , Interferon-γ (IFN-γ) with a single sample testing.

Human Th1/Th2/Th17 Cytokine Panel (Flow Cytometry Multiplex Beads Assay) gives quantitative results of Interleukin-2 (IL-2), Interleukin-4 (IL-4), Interleukin-6 (IL-6), Interleukin-10 (IL-10), Tumor Necrosis Factor α type (TNF-α) , Interferon-γ (IFN-γ) and Interleukin-17A (IL-17A) with a single sample testing.

The performance of this panel has been optimized for specific analysis of cytokines in cell culture supernatants, plasma and serum samples. The kit provides sufficient reagents for the quantitative analysis of 96 tests.

We offer a range of individual reagents for each factor, giving you the flexibility to select and combine them according to your specific research requirements.

【Background】

Cytokine plays an important role in physiological and pathological processes such as immune regulation, inflammatory response and tumor metastasis. Cytokine detection was wildly used in infectious disease, autoimmune disease, tumor auxiliary diagnosis, disease evaluation, medication guidance and prognosis judgment.

Multiple cytokines can be detected by the flow cytometry multiplex bead assay at the same time, and multiple results can be obtained in a single sample test at once time, which would reduce the costs of samples and reagents. It could be used in kinds of flow cytometers. The assay is easy to operate.

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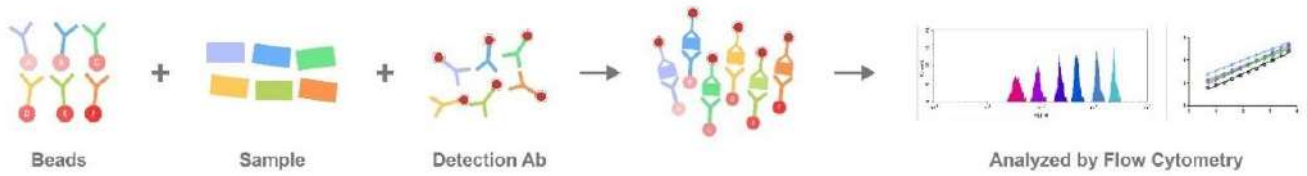
【Principle】

The capture beads have been conjugated with the specific antibody, different beads have known fluorescence which would be possible to detect respectively. The detection reagent is a mixture of phycoerythrin (PE)-conjugated antibodies. The capture beads, proteins (IL-2/4/6/10, TNF-α, IFN-γ, IL-17A) and detection antibodies formed a sandwich complex as bead-analyte-detection antibody. The intensity of PE fluorescence, in proportion to the recombinant protein titer.



【Product】

Table 1. Recommended Products For 96 Tests

Product	Cat. No.	Size	Format	Storage (Unopened)	Storage (Opened)
Anti-human IL-2 Antibody-coupled APC-beads-A06	MBS-K078	96 tests	Beads suspension	2-8 ℃, avoid light	2-8 ℃, avoid light
Anti-human IL-4 Antibody-coupled APC-beads-A05	MBS-K069	96 tests	Beads suspension	2-8 ℃, avoid light	2-8 ℃, avoid light
Anti-human IL-6 Antibody-coupled APC-beads-A04	MBS-K070	96 tests	Beads suspension	2-8 ℃, avoid light	2-8 ℃, avoid light
Anti-human IL-10 Antibody-coupled APC-beads-A03	MBS-K071	96 tests	Beads suspension	2-8 ℃, avoid light	2-8 ℃, avoid light
Anti-human TNF-alpha Antibody-coupled APC-beads-A02	MBS-K072	96 tests	Beads suspension	2-8 ℃, avoid light	2-8 ℃, avoid light
Anti-human IFN-gamma Antibody-coupled APC-beads-A01	MBS-K073	96 tests	Beads suspension	2-8 ℃, avoid light	2-8 ℃, avoid light
Anti-human IL-17A Antibody-coupled APC-beads-A07	MBS-K081	96 tests	Beads suspension	2-8 ℃, avoid light	2-8 ℃, avoid light
Th1/Th2 Standard or	IL2-H51P3/ IL2-H51P4	5 μg	Powder	2-8 ℃	-20 ℃

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Th1/Th2/Th17 Standard					
PE-Labeled Detection Antibody-1 or PE-Labeled Detection Antibody-2	FABm010-01/ FABm013-01	0.5 mL	Liquid	2-8 ℃, avoid light	2-8 ℃, avoid light
2 × Assay Buffer	FCMB-01	40 mL	Liquid	2-8 ℃	2-8 ℃
10 × Wash Buffer	FCMB-02	10 mL	Liquid	2-8 ℃	2-8 ℃
96-Well V-Bottom Assay Plate	FCMP-01	1 plate	Solid	RT	RT
96-Well Sealing Film	FCMP-02	2 pieces	Solid	RT	RT
PE Positive Control	MBS-K075	0.5 mL	Beads suspension	2-8 ℃, avoid light	2-8 ℃, avoid light
APC Positive Control	MBS-K074	0.5 mL	Beads suspension	2-8 ℃, avoid light	2-8 ℃, avoid light

【Unsupplied Materials and Instruments】

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 separation rack (Magnetic Capture Plate, catalog FCM-C03M)
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

【Storage and Expiration】

1. Unopened kit should be stored at 2 ℃~8 ℃ upon receiving.
2. The expiration date is labeled on the package box. DO NOT use reagents beyond expiration date.
3. 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 (Cat. IL2-H51P3 or IL2-H51P4) is*

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reconstituted.

【Important】

1. For research use only, not for use in diagnostic or therapeutic procedures.
2. Please follow the instructions strictly, for optimal and consistent data output.
3. Protect beads suspension, detection antibody from light all times to prevent photobleaching.
4. DO NOT mix or substitute reagents from different kit lots. DO NOT mix up or substitute reagents from different manufacturers.
5. Bring the kit components to room temperature before use. Be sure the crystal precipitates are all dissolved before use.
6. Prepare the buffer, reagents, calibrator, samples and all relevance, just prior to use.
7. Deionized or distilled water must be used for reagent preparation.
8. Ensure reagent reservoirs are clean.
9. In order to avoid microbial contamination or cross-contamination of reagents or specimens which may invalidate the test, use disposable plastic pipette tips.
10. Avoid long-term storage and repeated freeze-thaw cycles of reconstituted calibrator.

【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 guideline.

【Procedure】

1. Assay Preparation

1.1 Preparation of Samples

1.1.1 Cell culture supernatant

Fresh collected or -80 ℃ stored medium 4000 g centrifuge for 10 minutes at 4 ℃, aspirate the supernatant and used for the next assay.

1.1.2 Serum collection and storage

1.1.2.1 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 ℃ (Excessive centrifugation might lead to hemolysis). Aspirate the serum layer and avoid the contamination of blood cells.

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

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

1.1.3 Plasma collection and storage

1.1.3.1 Fresh blood samples were obtained from venous, adding anticoagulant sodium citrate, such as EDTA or heparin. 2, 000 g centrifuge for 10 minutes at 4 ℃. Carefully aspirate the plasma layer, and avoid the contamination of blood cells.

1.1.3.2 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, 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.*

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

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1.2 Preparation of Reagents and Buffer

For a repeatable detection assay, we recommend bring the kit to room temperature and keep 15 minutes before use for a temperature balancing. The following preparation steps are intended for 96 tests assay.

1.2.1 Assay Buffer Working Solution

Aspirate 40 mL 2 × Assay Buffer (Cat.FCMB-01), mixed with 40 mL deionized water.

Note: *Assay Buffer Working Solution is used for preparation of calibrator, samples and detection antibody.*

1.2.2 Wash Buffer Working Solution

Aspirate 10 mL 10 × Wash Buffer (Cat. FCMB-02), mixed with 90 mL deionized water.

Note: *Wash Buffer Working Solution is used for reaction product washing and beads resuspended.*

1.2.3 Detection Antibody Working Solution

Aspirate 0.5 mL PE-Labeled Detection antibody (Cat. FABm013-01 or FABm010-01), mixed with 10.5 mL Assay Buffer Working Solution.

Note 1: *Select Cat. FABm013-01 if IL-17A was chosen to detetct.*

Note 2: *The preparation of Detection Antibody Working Solution can be regulated according to samples and a freshly prepared Detection Antibody Working Solution is recommend for a better performance.*

1.2.4 Beads Suspension Working Solution

Select Antibody-coupled APC-beads according to your demand (from Cat. MBS-K069 / MBS-K070 / MBS-K071 / MBS-K072 / MBS-K073 / MBS-K079 / MBS-K081). 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 Working Solution.

We recommend a freshly prepared reagents for usage of the beads. Each test requires total volume of 120 μL Beads Suspension Working Solution. Multiplex assay should be mixed

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and diluted as indicated in Table 2.

Table 2. Preparation of Beads Suspension Working Solution

	1 Test	N Tests
Antibody-coupled APC-beads	1 μL for each beads	N*1 μL for each beads
Assay Buffer	(120-M*1) μL	N*(120-M*1) μL

Note: M is the number of detection targets, N is the number of tests.

1.3 Preparation of Calibrator

1.3.1 Reconstitute lyophilized Th1/Th2/Th17 Standard (Cat. IL2-H51P4 or IL2-H51P3) powder with 500 μL deionized water, as calibrator stock#1 with each analyte 10 μg/mL respectively. For completely dissolving, keep the bottle at room temperature at least for 15 minutes.

Note 1: *Select Cat. IL2-H51P4 if IL-17A was chosen to detetct.*

Note 2: *Mix or reconstitute protein reagent gently, avoid bubbles and foam.*

1.3.2 Add in 900 μL Assay Buffer Working Solution in a new tube, aspirate 100 uL calibrator stock#1, labeled as calibrator stock#2. The concentration of each analyte is 1 μg/mL respectively.

1.3.3 Repeat operation of step 1.3.2 and labeled as stock#3 and Cal 11 respectively.

1.3.4 Performing 2-fold serial dilutions from Cal 11, add 500 μL Assay Buffer Working Solution, labeled as Cal 10, Cal 9, Cal 8, Cal 7, Cal 6, Cal 5, Cal 4, Cal 3, Cal 2 and Cal 1 respectively, as shown in Table 3.

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

Table 3. Preparation of Calibrator

Calibrator ID	Serial Dilution	Assay Buffer Working Solution add in (μL)	Calibrator add in (μL)	Final Concentration (pg/mL)
Stock#2	10	900	100 μL of stock#1	1,000,000
Stock#3	10	900	100 μL of stock#2	100,000
Cal 11	10	900	100 μL of stock#3	10,000

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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 Samples preparation. Dilute 30 µL freshly prepared or thawed plasma / serum / cell culture supernatant with 30 µL Assay Buffer Working Solution, mix homogenously and ready for being used.

Note 1: *Only 30 µL dilute sample needed per well for this assay, please calculate appropriate dilution volume.*

Note 2: *Samples dilution could be affected by the concentration of the cytokines and matrix effect.*

2.2 Add serial dilutions of Calibrator or samples to 96-well V-bottom plate, 30 µL per well.

Note: *We recommended to run calibrators in duplicates for good accuracy.*

2.3 Add Beads Suspension Working Solution to 96-Well V-Bottom Assay Plate, 120 µL per well.

2.4 Seal the plate. Incubate at room temperature for 120 minutes, with continuous shaking 400-600 rpm. Avoid light.

Note: *Please adjust the shaking speed to ensure the beads always suspended homogenously in the solution.*

2.5 Place the plate onto the magnetic separation rack for 2 minutes, carefully discard supernatant (avoid pipetting beads).

Note 1:*Please take a few seconds to minutes longer if the separation was insufficient.*

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Note 2: *If the magnetic separation rack was unavailable, please use centrifuge to separate beads, RCF 500g~1000g, 5 min at room temperature is recommended.*

2.6 Remove the plate from separation rack, add 200 µL Wash Buffer Working Solution.

Note: *We recommend to use pipette to aspirate and dispense 2~3 times to make sure the beads be washed well.*

2.7 Place the plate onto the magnetic separation rack for 2 minutes， carefully discard supernatant (avoid pipetting beads).

2.8 Remove the plate from separation rack. Add Detection Antibody Working Solution, 100 µL per well.

2.9 Seal the plate. Incubate at room temperature for 60 minutes, with continuous shaking 400-600 rpm to ensure the beads always suspended homogenously in the solution. Avoid light.

Note: *Please adjust the shaking speed to ensure the beads always suspended homogenously in the solution.*

2.10 Place the plate onto the magnetic separation rack for 2 minutes, carefully discard supernatant (avoid pipetting beads).

2.11 Repeat step 2.5, 2.6 and 2.7

Note: *The washing step can be repeated one more time for reducing the noise level.*

2.12 Remove the plate from separation rack. Add 150 µL Wash Buffer Working Solution to each well. Mix by pipetting up and down. Ensure the beads well separated and not aggregated.

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

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

3. Flow Cytometer Setup

3.1 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.

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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

3.2 Channel setup

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

3.2.2 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.

3.3 Select medium flow rate.

3.4 Set up 500 events or beads per plex (3,000 events for 6 plex) collected in P1 gate as stop criteria.

4. Data Acquisition and Analysis

4.1 Data acquisition

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

4.1.2 Create an experiment in 96-well plate format.

Note: *If 96-well plate loader is not available, transfer the samples to FACS tubes, replenish with 100 µL Wash Buffer Working Solution, and read one by one.*

4.1.3 Resuspend beads by pipetting up and down.

4.1.4 Load the plate and start acquisition.

4.1.5 In APC-count histogram, create P2 ~ P7 gates in P1 sub-population. Six-plex beads was

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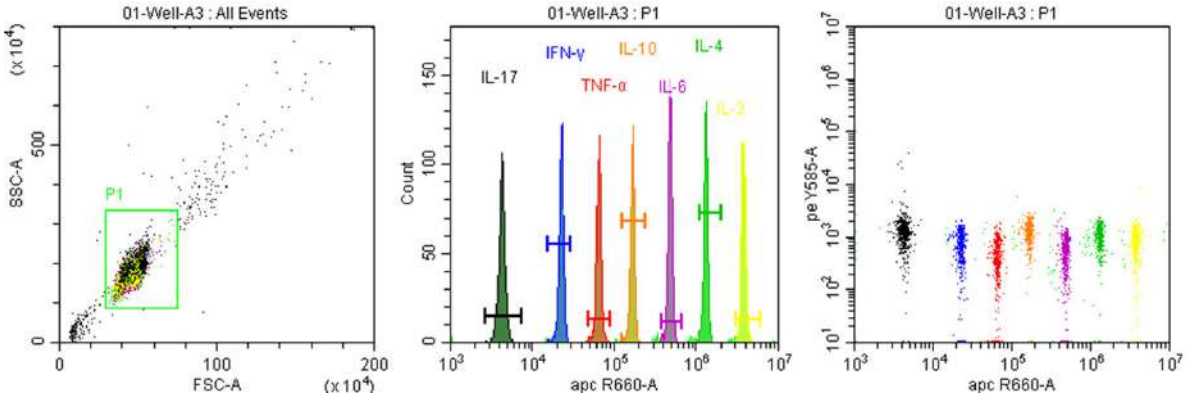
differentiated, as indicated in Figure 1.

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

Figure 1. Standards of Analyte-specific Populations



4.2 Data analysis

4.2.1 The following data, acquired by BECKMAN Cytoflex, as indicated in Table 5.

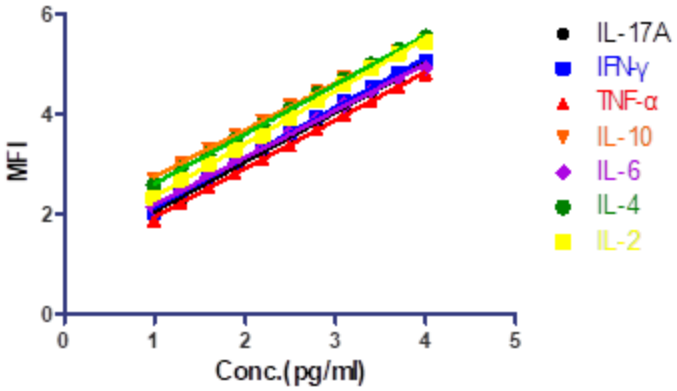
4.2.2 Two-log-linear fit curve model is applied by data analysis with GraphPad by plotting Log10 concentration value of serial diluted calibrators against Log10 median fluorescence intensity (MFI) of PE channel. We recommend the r^2 value of the curve above 0.99.

Note: *Please substract the MFI of 0 concentration from every concentration.*

4.2.3 Standard curve examples, the following graphs represent standard curves from the Human Th1/Th2 Cytokine Panel (Flow Cytometry Multiplex Bead Assay), as indicated in Figure 2.

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

Figure 2. Standard Curves



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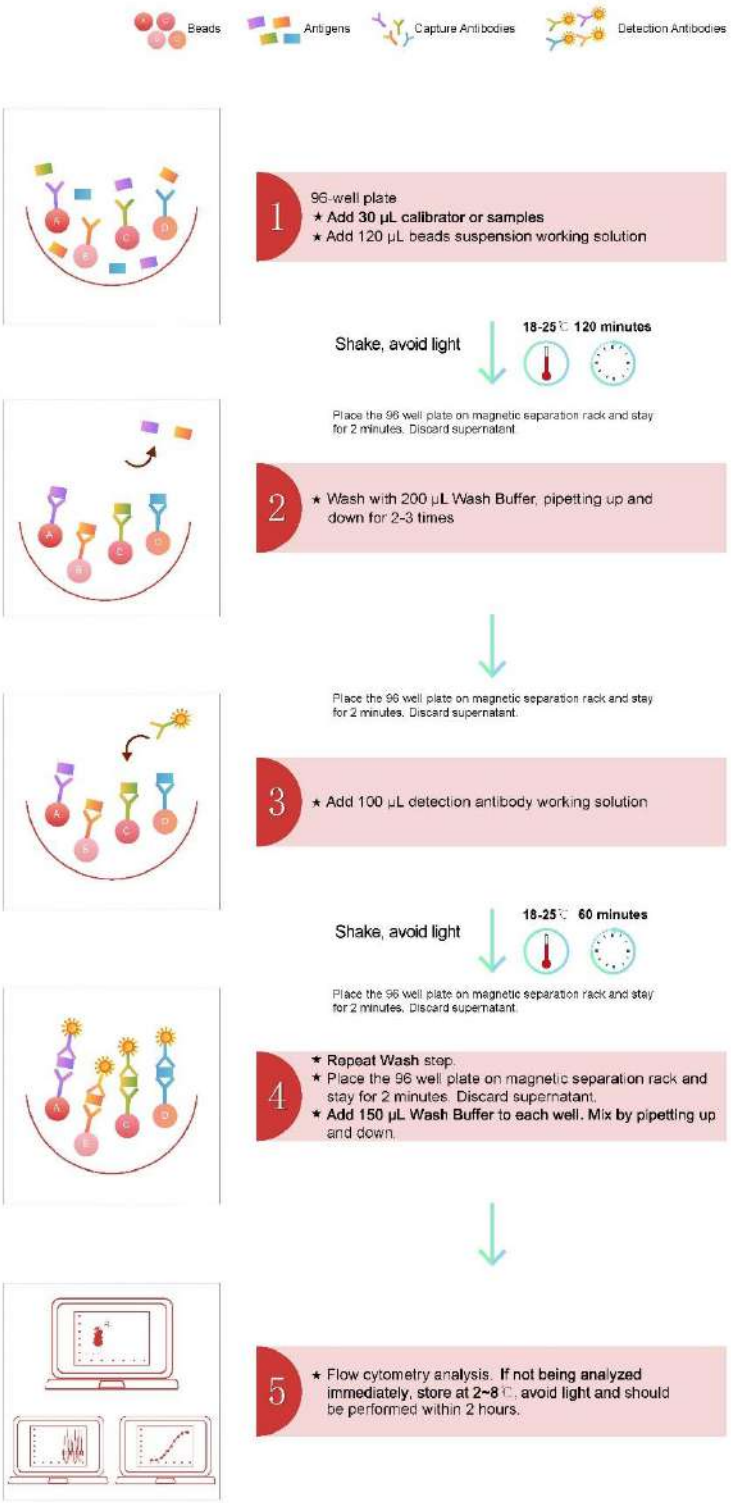
【Quick Guide】



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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 beads 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 6 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 6 intact peaks observed.
Less than 6 bead populations in APC-PE scatter plot, though all the 6 peaks obtained in APC-count histogram.	The PMT gain or voltage value of PE fluorescent channel is too high.	Adjust PMT gain or voltage of PE fluorescent channel, ensuring 6 bead populations in APC-PE scatter plot.

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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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