SARS-CoV-2 Variants Neutralizing Antibody 6-plex Panel

(Flow Cytometry Multiplex Bead Assay)

【Product Name】

SARS-CoV-2 Variants Neutralizing Antibody 6-plex Panel (Flow Cytometry Multiplex Bead Assay)

【Catalog】

FCM-N04R

【Size】

96 Tests

【Background】

Since December 2019, the emergence of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) and its associated disease, COVID-19, has caused a devastating pandemic worldwide. SARS-CoV-2 is a single stranded RNA virus, composed of four structural proteins, that are Spike (S) protein, Envelop (E) protein, Nucleocapsid (N) protein, and Membrane (M) protein. The viral Spike protein binds the host receptor angiotensin-converting enzyme 2 (ACE2) via the receptor binding domain (RBD), which is believed to be the mechanism that SARS-CoV-2 invades mammalian cells. SARS-CoV-2 continuously evolves, triggering pandemic waves one after another in the world. Several variants have been defined as Variant of Concern (VOC), evoking increased transmissibility and disease severity, for instance, Alpha (B.1.1.7), Beta (B.1.351), Gamma (P.1), Delta (B.1.617.2), Omicron (B.1.1.529).

Flow cytometry is originally an instrument to analyze single-cell properties in cell suspension. Nowadays, the application of flow cytometry has been extended to bead-based binding assays. Flow Cytometry Multiplex Bead Assay allows for rapid and sensitive profiling of several analytes in high-throughput format. This approach assesses dozens of analytes in a single reaction, which can generate more data with less specimen, reduced labor, and lower cost per analyte. Flow Cytometry Multiplex Bead Assay provided by ACROBiosystems are vigorously validated to ensure the test results are precise and accurate, performing robustly in supporting drug development and other intended purpose.

In principle, magnetic or non-magnetic beads with diameter 4 ~ 6 μm, were colored with infrared or red fluorescent dyes, differentiated regionally by size, dye intensity, or fluorescent spectrum on flow cytometer. Each fluorescent microsphere population is conjugated to a specific target analyte, then mixed according to panel design. Processing an immunoassay in bead suspension, binding signal from bead events are acquired and statistically analyzed by flow cytometry. A common flow cytometer equipped with blue and red lasers are sufficient to carry out multiplex immunoassay, not requiring installation of delicate instruments.

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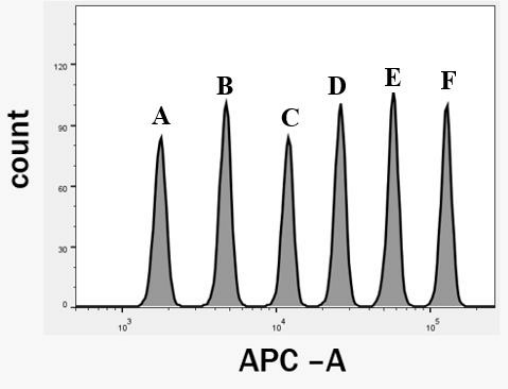
The kit, **ACROBiosystems cat#FCM-N04R**, is designed for simultaneous detection of neutralizing antibodies to six SARS-CoV-2 variants (Wild-type, Alpha | B.1.1.7, Beta | B.1.351, Gamma | P.1, Delta | B.1.617.2, Omicron | B.1.1.529) , in a single human serum or plasma specimen.

This product is for research use only, not for use in diagnostic or therapeutic procedures.

【Principle】

SARS-CoV-2 Variants Neutralizing Antibody 6-plex Panel is a multiplex bead-based competitive immunofluorescence assay, utilizing flow cytometry for data acquisition and classification. Six SARS-CoV-2 Spike protein (WT / Alpha / Beta / Gamma / Delta / Omicron) were conjugated to 6 fluorochrome-encoded microspheres respectively, indicated in Table 1. Anti-SARS-CoV-2 neutralizing antibody in blood specimens competes with human ACE2, for binding to Spike protein encapsulated 6-plex beads. Streptavidin conjugated R-phycoerythrin (SA-PE) was employed to detect biotinylated human ACE2 bound to the beads. The intensity of PE fluorescence was assessed by flow cytometry at wavelength of 575 nm approximately, in inversely proportion to the Anti-SARS-CoV-2 neutralizing antibody in blood specimens. The intensity of APC fluorescence was applied to classify bead populations, at wavelength of 670 nm approximately.

Table 1. Analytes and Beads



Beads	Variant of Concern
A	WT (Wild-type)
B	Alpha | B.1.1.7
C	Beta | B.1.351
D	Gamma | P.1
E	Delta | B.1.617.2
F	Omicron | B.1.1.529

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【Components】

Table 2. Material provided and storage condition

ID	Components	Size (96 tests)	Format	Storage (Unopened)	Storage (Opened)
FCM04-C01	SARS-CoV-2 Antibody Calibrator	20 μg	Powder	2-8°C	-20°C
FCM04-C02	RBD 6-plex Beads	200 μL	Beads suspension	2-8°C Light-sensitive	2-8°C Light-sensitive
FCM04-C03	Assay Buffer	55 mL	Liquid	2-8°C	2-8°C
FCM04-C04	5 × Wash Buffer	10 mL	Liquid	2-8°C	2-8°C
FCM04-C05	Biotinylated Human ACE2	40 μg	Powder	2-8°C	-20°C
FCM04-C06	5 × PBS Buffer	10 mL	Liquid	2-8°C	2-8°C
FCM04-C07	APC Beads	500 μL	Beads suspension	2-8°C Light-sensitive	2-8°C Light-sensitive
FCM04-C08	PE Beads	500 μL	Beads suspension	2-8°C Light-sensitive	2-8°C Light-sensitive
FCM04-C09	96-Well V-bottom Plate	1 plate	/	2-8°C or RT	2-8°C or RT
FCM04-C10	96-Well Sealing Film	2 pieces	/	2-8°C or RT	2-8°C or RT
FCM04-C11	SA-PE	200 μL	Liquid	2-8°C Light-sensitive	2-8°C Light-sensitive

【Shipping and Storage】

1. **Unopened:** Store at 2°C to 8°C upon receipt. The expiration date is labeled on the box.
2. **Opened:** The opened kit should be stored per components table. The shelf life is 30 days from the date of opening. Do not use reagents beyond expiration date.

Note: *Freeze and thaw NO MORE THAN 2 times, once calibrator (ID# FCM04-C01) or biotinylated human ACE2 (ID# FCM04-C05) is reconstituted.*

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【Unsupplied Material and Instrument】

1. Single-channel pipettes, multi-channel pipettes, and pipette tips
2. Reagent reservoirs for multi-channel 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

【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, SA-PE from light all times to prevent photobleaching.
4. DON'T mix or substitute reagents from different kit lots. DON'T 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, reagent, calibrator, sample 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 and reconstituted biotinylated human ACE2.

【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 that it is used in accordance with the principles of good laboratory practice. Wear suitable protective clothing such as laboratory overalls, safety glasses and gloves. Care should be taken 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.

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【Procedure】

1. Preparation of sample and reagent

1.1 Serum collection and storage

1.1.1 Fresh blood samples were obtained from venous, standing at room temperature more than 30 minutes. After coagulation, centrifuge 10 minutes at 2000g, 4℃ (Excessive centrifugation might lead to hemolysis). Carefully aspirate the serum layer, and avoid the contamination with blood cells.

1.1.2 Centrifuge the serum layer for 10 minutes, at 16000g, 4℃. Discard the precipitates, and keep supernatant as the freshly prepared blood serum. Use the serum immediately or keep the serum at -80℃ freezer.

1.1.3 Dilute 10 μL freshly prepared or thawed serum in 50 μL Assay Buffer, mix homogenously. Serum sample is ready for being tested.

1.2 Plasma collection and storage

1.2.1 Fresh blood samples were obtained from venous, adding anticoagulant sodium citrate, EDTA, or heparin. Centrifuge 10 minutes at 2000g, 4℃. Carefully aspirate the plasma layer, and avoid the contamination with blood cells.

1.2.2 Centrifuge the plasma layer 10 minutes, at 16000g, 4℃. Discard the precipitates, and keep supernatant as the freshly prepared blood plasma. Use the plasma immediately or keep the plasma at -80℃ freezer.

1.2.3 Dilute 10 μL freshly prepared or thawed plasma in 50 μL Assay Buffer, mix homogenously. Plasma sample is ready for being tested.

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*.

1.3 Preparation of calibrator

1.3.1 Reconstitute 20 μg Calibrator powder in 480.7 μL Assay Buffer. Allow the bottle to sit at room temperature for 15 minutes, for completely dissolving. This is calibrator stock.

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

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1.3.2 In a microcentrifuge tube, add 180 µL Assay Buffer, 120 µL calibrator stock, and mix well. Labeled as Cal9.

1.3.3 In microcentrifuge tubes, add 150 µL Assay Buffer, labeled as Cal8, Cal7, Cal6, Cal5, Cal4, Cal3, Cal2 and Cal1, respectively.

1.3.4 Perform 2-fold serial dilutions from Cal9, as showed in Table 3.

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

Table 3. Calibrator

Calibrator ID	Serial Dilution	Assay Buffer to add (µL)	Calibrator to add (µL)	Final Concentration (ng/mL)
Cal9	-	180	120 µL of Stock	16640
Cal8	2	150	150 µL of Cal9	8320
Cal7	2	150	150 µL of Cal8	4160
Cal6	2	150	150 µL of Cal7	2080
Cal5	2	150	150 µL of Cal6	1040
Cal4	2	150	150 µL of Cal5	520
Cal3	2	150	150 µL of Cal4	260
Cal2	2	150	150 µL of Cal3	130
Cal1	2	150	150 µL of Cal2	65

1.4 Preparation of reagent and buffer

1.4.1 Beads suspension working solution：Vortex RBD 6-plex Beads suspension vigorously for 30 seconds or more. Immediately transfer 200 µL RBD 6-plex Beads to a microcentrifuge tube, mixed with 17.4 mL Assay Buffer.

Note: *Magnetic beads might aggregate during long-time storage. If beads aggregates observed during liquid transferring, vortex the beads once again until aggregates are invisiable.*

1.4.2 Biotinylated Human ACE2 working solution：Reconstitute 40 µg Biotinylated Human

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ACE2 powder in 1 mL Assay Buffer. Allow the bottle to sit at room temperature for 15 minutes, for completely dissolving. Transfer dissolved ACE2 solution to a new tube. Rinse the original bottle with 3 mL Assay Buffer and combine with the previous one, to prepare 4 mL biotinylated human ACE2 working solution.

1.4.3 1 × wash buffer：Bring 5 × Wash Buffer to room temperature. Aspirate 10 mL 5 × Wash Buffer, mixed with 40 mL ultrapure water.

1.4.4 1 × PBS：Bring 5 × PBS Buffer to room temperature. Aspirate 10 mL 5 × PBS Buffer, mixed with 40 ml ultrapure water.

1.4.5 SA-PE working solution：Bring SA-PE to room temperature. Aspirate 200 µL SA-PE, mixed with 3.8 mL Assay Buffer.

2. Assay procedure

2.1 Add serial dilutions of calibrator, or properly diluted blood 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.*

2.2 Add beads suspension working solution to 96-well plate, 160 µL per well.

2.3 Add biotinylated human ACE2 working solution to 96-well plate, 30 µL per well.

Note： *Add samples, beads suspension, and biotinylated human ACE2 sequentially.*

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

Note: *Wrap the 96-well plate by aluminum foil, or keep orbital shaker in the dark.*

2.5 Peel off the Sealing film, and DO NOT discard the supernatant. Add SA-PE working solution to 96-well plate, 30 µL per well.

2.6 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.

2.7 Place the 96-well plate on magnetic separation rack and stay for 2 minutes. Discard supernatant.

Note： *Magnets vary in strength. It may take a few seconds to minutes to complete the separation.*

2.8 Remove the plate from magnetic separation rack. Add 200 µL 1 × wash buffer to each well and suspend the beads.

2.9 Place the 96-well plate on magnetic separation rack and stay for 2 minutes. Discard supernatant.

2.10 Remove the plate from magnetic separation rack. Add 200 µL 1 × PBS to each well, mixed by pipetting up and down. Ensure the beads well separated and not aggregated.

2.11 Subject to flow cytometry analysis. If not being analyzed immediately, store at 2 ~ 8℃ and protect from light. Read by flow cytometry within 2 hours.

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

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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. Instruments tested by this assay were represented in Table 4.

Table 4. Partial list of compatible flow cytometers

Manufacturer	Flow Cytometer	Classification Channel	Reporter Channel
BD Biosciences	BD FACSLyric™	APC	PE
Beckman Coulter	Cytoflex S	APC	PE
Thermo Fisher Scientific	Attune NxT	RL1	YL1
Luminex Corporation	Guava easyCyte3L	RED-R	YEL-B

3.2 Start up and calibrate flow cytometer following the instrument user guide.

3.3 Enable FSC and SSC channel.

3.4 Enable the fluorescent channel with an excitation peak at 488 nm or 532 nm, and an emission peak around 575 nm (for example, PE channel). Enable the fluorescent channel with an excitation peak at 633 nm, and an emission peak around 670 nm (for example, APC channel).

3.5 Load APC Beads, and adjust PMT gain or voltage of FSC and SSC, positioning the singlet beads in the central region of FSC-SSC scatter plot. Adjust FSC and SSC threshold to diminish debris.

3.6 In FSC-SSC scatter plot, draw a region around the single beads to exclude doublets or more, and gate all subsequent analysis on this region, labeled as P1.

3.7 In APC-count histogram, P1 sub-population represent two peaks. Adjust gain or voltage of APC channel, positioning APC positive peak close to upper limit of APC scale, APC negative peak between 0 ~ 1000 correspondingly.

3.8 Remove APC Beads and load PE Beads. In FSC-SSC scatter plot, draw P1 gate in the same way as above. In PE-count histogram, P1 sub-population represents two peaks. Adjust gain or voltage of PE channel, positioning PE positive peak close to upper limit of PE scale, PE negative peak between 0 ~ 1000 correspondingly. Thus, the gain or voltage are configurated.

3.9 Load APC Beads and PE Beads once again to adjust fluorescence spillover of APC and PE channel, following the instrument user guide or former experiences to establish compensation matrix. (In most instances, APC doesn't spillover into PE channel, and vice versa).

3.10 Select medium flow rate.

3.11 Set up stop criteria as 4800 events or beads collected in P1 gate.

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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 100 µL 1× PBS, and read one by one.*

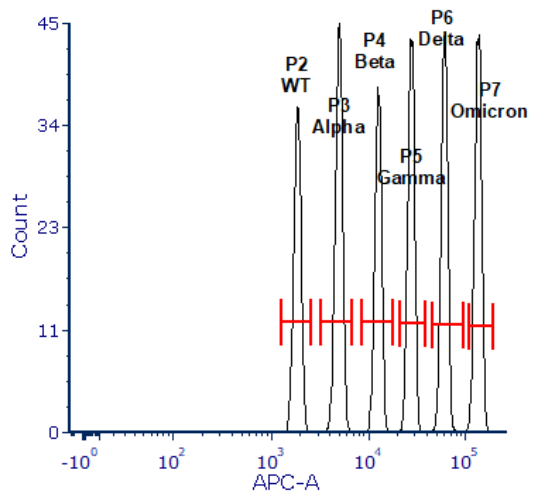
4.1.3 Resuspend beads by pipetting up and down.

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 RBD beads was differentiated, indicated in Table 5.

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

Table 5. Segregation of analyte-specific populations



Beads	Variant of Concern	Gate
A	WT (Wild-type)	P2
B	Alpha | B.1.1.7	P3
C	Beta | B.1.351	P4
D	Gamma | P.1	P5
E	Delta | B.1.617.2	P6
F	Omicron | B.1.1.529	P7

4.2 Data analysis

4.2.1 Construct a calibration curve for each analyte by plotting Log_{10} concentration value of serial diluted calibrators against median fluorescence intensity (MFI) of PE channel. A four parameter logistic (4-PL) curve model is applied, with R^2 value above 0.99.

4.2.2 Calculate the concentration of unknown from the calibrator curve of each analyte.

4.2.3 Determine the concentration of unknown in blood specimens after multiplying by the dilution factor (6×).

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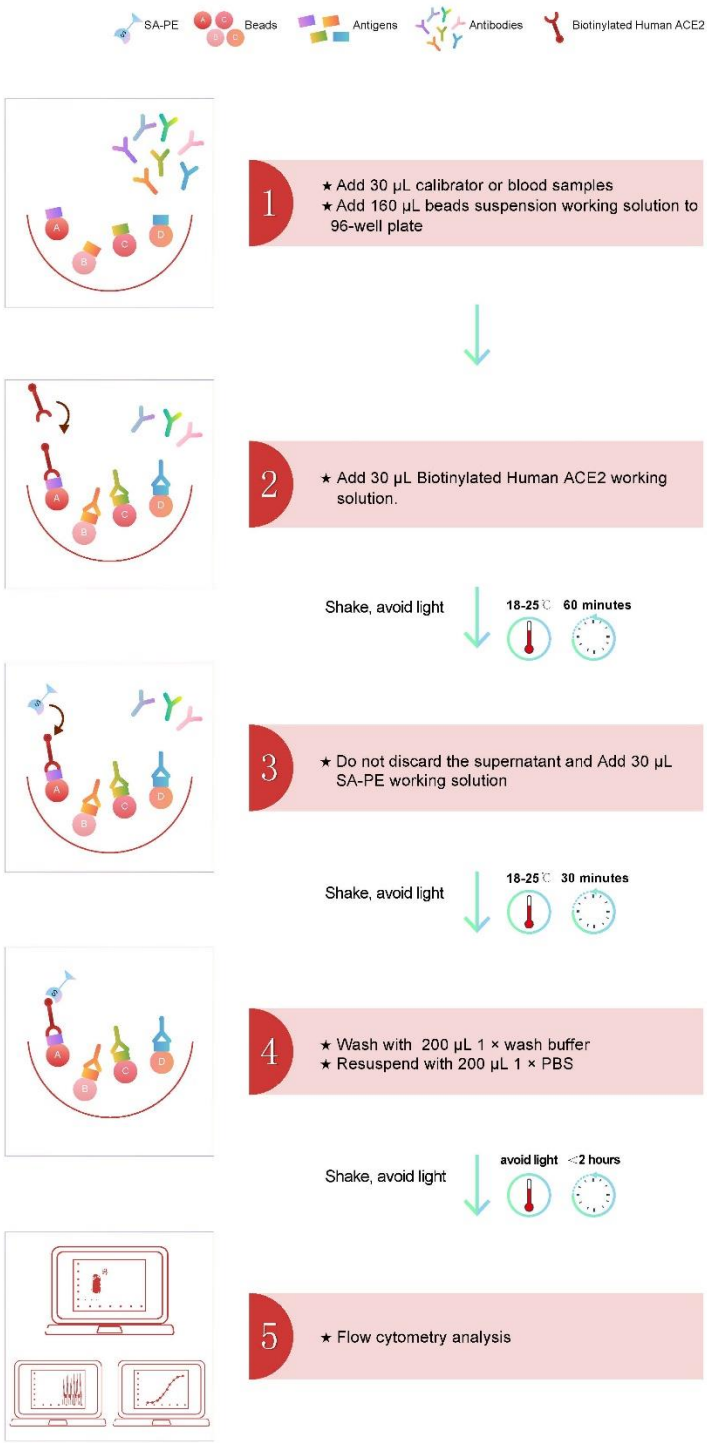
【Quick Guide】

Print the Quick Guide and follow the protocol.



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【Troubleshooting】

Concerns	Possibilities	Suggestions
After magnetic separation, the brown bead precipitates are not visible or become less and less after multiple-step operation.	Magnets vary in strength. Incomplete separation leads to loss of beads.	Replace with stronger magnets, or stay on the magnetic rack for longer time for binding to occur.
Variation of bead count in duplicated wells.	Aspiration takes too long time, that the beads settled to the bottom of the tube.	Fast aspiration and dispensing of the bead suspension. Vortex beads vigorously before first use, and vortex briefly in between.
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 were 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.
PE fluorescent intensity of low concentration calibrator is lower than that of high concentration calibrator.	Insufficient needle wash and clean between samples.	At least one washing cycle between samples in flow cytometry setting.
		Follow the Plate Layout suggested, and read the plate by columns to reduce cross-contamination on flow cytometer.

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【Plate Layout】

Arrange the samples in vertical, and read the plate by column.

	1	2	3	4	5	6	7	8	9	10	11	12
A	Cal9	Cal5	Cal1	.....								
B	Cal9	Cal5	Cal1	.....								
C	Cal8	Cal4	Sample	.....								
D	Cal8	Cal4	Sample	.....								
E	Cal7	Cal3	Sample	.....								
F	Cal7	Cal3	Sample	.....								
G	Cal6	Cal2	Sample	.....								
H	Cal6	Cal2	Sample	.....								

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