Mouse Cell Release Factor Detection by Flow Cytometry Multiplex Bead Assay

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】

Multiplex Bead Assay platform allows flow cytometry based on beads with different size and fluorescence intensity to quantity multiple soluble cell release factor from a single sample. The performance of this platform has been optimized for specific analysis in cell culture supernatants, plasma and serum samples. Compared to traditional single-analyte methods, it offers significant advantages, including lower sample volume requirements, simpler operation, more broad dynamic range and so on.

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.

【Principle】

The antibody coupled beads could be differentiated by different fluorescence intensities and size, which made the capture beads form different sets. Each bead set have been conjugated with the specific antibody for the particular analyte. The target analytes specific bind to the capture antibodies, which were coupled to the beads. Then biotinylated detection antibodies are added, each biotinylated detection antibody bind to specific analyte. Thus, the capture beads, analyte and detection antibodies formed a sandwich complex as bead-analyte-detection antibody. Streptavidin-phycoerythrin (SA-PE) is subsequently added and would bind to biotinylated detection antibodies. The intensity of PE fluorescence is in proportion to the concentration of the analyte. The principle is shown as Figure 1.

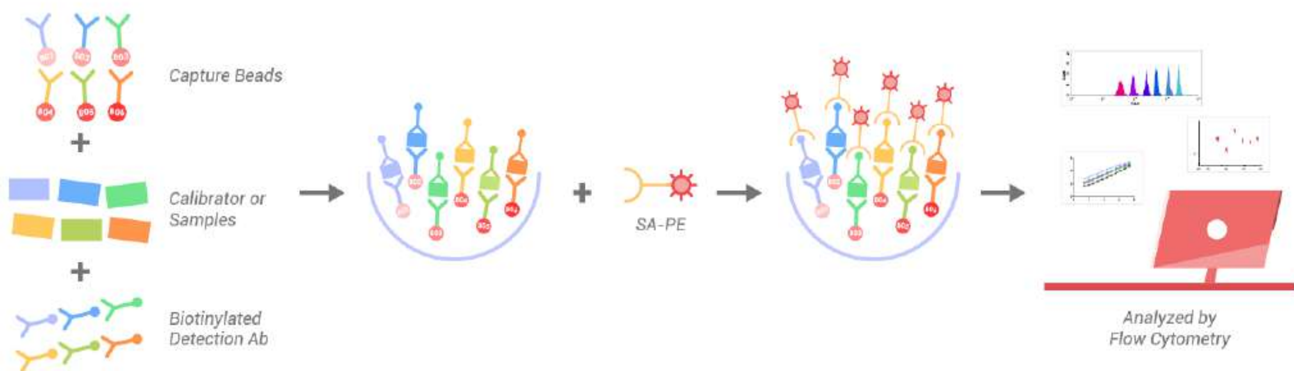

Figure 1. Principle of Multiplex Bead Assay

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There are many sets of beads but with one size based on their forward scatter (FSC) and side scatter (SSC) profiles as P1 (Figure 2). Each set has a unique fluorescence intensity, which can be identified by APC channel (Figure 3).

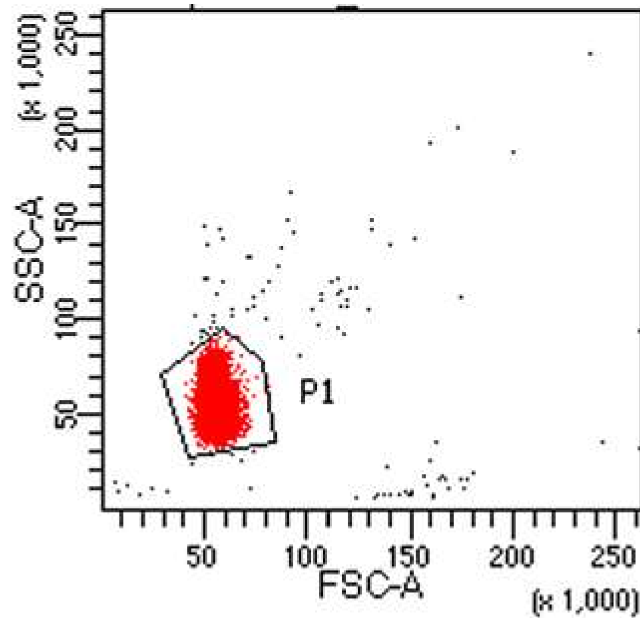

Figure 2. Gate of Capture Beads

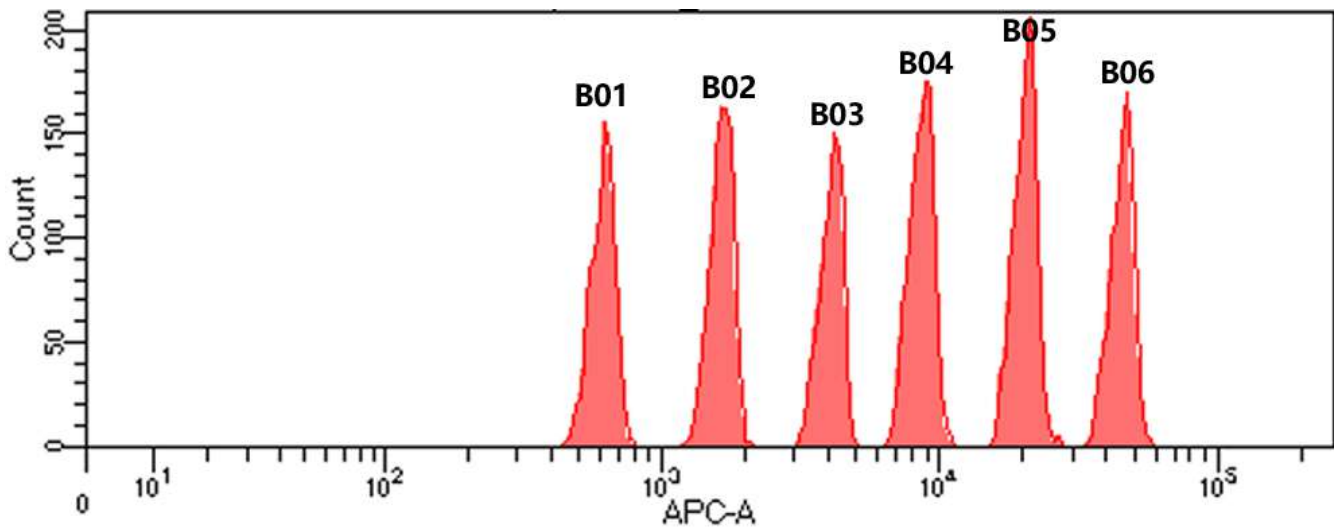

Figure 3. Beads Classification

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【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. Horizontal orbital shaker for 96-well plate
6. Vortex mixer
7. 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, 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.

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【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.

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【Procedure】

1. Assay Preparation

1.1 Preparation of Samples

1.1.1 Mouse serum

Fresh mouse serum could be subject to assay directly or diluted with Assay Buffer according to estimated concentration

Frozen serum should be mixed thoroughly after thawing, and centrifuged at 16000g 10min to remove all visible debris.

Note: *Thawed samples should be assayed immediately. Avoid repeated freeze-thaw cycles.*

1.2 Preparation of Reagents and Buffer

1.2.1 Capture Beads

Select Antibody-coupled APC-beads according to your demand. Vortex beads suspension vigorously no less than 30 seconds. Immediately transfer required volume of beads to a microcentrifuge tube according to Table 1, and dilute with Assay Buffer to obtain the Capture Beads. Each well requires 100 μL Capture Beads. Different sets can be multiplexd as indicated in Table 1.

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	(100-X*5) μL	Y*(100-X*5) μL

Note: *X is the number of plexes, Y is the number of wells.*

1.2.2 Detection Antibody

Select Biotinylated Antibody according to your demand. Dilute Biotinylated Antibody 20 times with Assay Buffer to obtain Detection Antibody. Each well requires 100 μL Detection Antibody. Different sets can be multiplexed as indicated in Table 2.

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Table 2. Preparation of Detection Antibody

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

Note: *X is the number of plexes, Y is the number of wells.*

1.2.3 SA-PE Solution

Reconstitute lyophilized Streptavidin Protein-PE powder in 200 μL deionized water at room temperature for 15 minutes with an initial concentration of 500 μg/mL. Dilute the reconstituted Streptavidin Protein-PE to 1.5 μg/mL with Assay Buffer as shown in Table 3. Each well requires 200 μL 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	200 μL	Y*200 μL

Note: *Y is the number of wells.*

1.3 Preparation of Calibrator

1.3.1 Please see Certificate of Analysis (CoA) for specific instructions. For best performance, we strongly recommend you to follow the reconstitution protocol provided in the CoA.

1.3.2 Dilute each reconstructed calibrator solution individually to 20 μg/mL with Assay Buffer, labeled as Stock#1 of each plex (ie. Stock#1 of mouse IL-2, Stock#1 of mouse IL-4…). The dilution volume for each reconstructed calibrator at different concentrations are shown in the Table 4.

Table 4. Preparation of Stock#1 of each plex

The Reconstructed Calibrator (μg/mL)	Volume of The Reconstructed Calibrator (μL)	Add Assay Buffer (μL)
200	30	270
400	15	285
500	12	288
600	10	290

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800	7.5	292.5
1000	6	294

1.3.3 Mix the individual Stock#1 of each plex into a single tube, labeled as Stock#2. Add Assay Buffer to make concentration of each plex 1 μg/mL in Stock#2. Different sets can be multiplexed as indicated in Table 5.

Table 5. Preparation of Stock#2

	1 Well	Y Wells
Stock#1	50 μL for each Stock#1	Y*50 μL for each Stock#1
Assay Buffer	(1000-X*50) μL	Y*(1000-X*50) μL

Note: *X is the number of plexes of Stock#1, Y is the number of wells.*

1.3.4 Add 900 μL Assay Buffer in a new tube, aspirate 100 μL Stock#2, labeled as Stock#3. The concentration is 0.1 μg/mL respectively.

1.3.5 Repeat step 1.3.4 and labeled as Cal 11.

1.3.6 Perform 2-fold serial dilutions from Cal 11, add 500 μL Assay Buffer, 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 6.

Table 6. Preparation of Calibrator

Calibrator ID	Serial Dilution	Assay Buffer Add in (μL)	Calibrator Add in (μL)	Final Concentration (pg/mL)
Stock#3	10	900	100 μL of Stock#2	100,000
Cal 11	10	900	100 μL of Stock#3	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 Add serial dilutions of Calibrator (Cal 0 - Cal 11) or samples to 96-well V-bottom plate, 30 μL per well.

2.2 Add Capture Beads to 96-well V-bottom plate, 100 μL per well.

2.3 Add Detection Antibody to 96-well V-bottom plate, 100 μL per well.

2.4 Seal the plate. Incubate at room temperature for 120 minutes, with continuous shaking 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.*

2.5 RCF 1000g centrifuge 5 minutes, discard supernatant and add 200 μL Wash Buffer.

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

2.6 Seal the plate. RCF 1000g centrifuge 5 minutes, discard supernatant and add 200 μL SA-PE Solution.

2.7 Seal the plate. Incubate at room temperature for 30 minutes, with continuous shaking 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.*

2.8 RCF 1000g centrifuge 5 minutes, discard supernatant and add 200 μL Wash Buffer.

Note 1: *We recommend to use pipette to aspirate and dispense 2~3 times to make sure the beads be washed well.*

Note 2: *The washing step can be repeated one more time for reducing the noise level.*

2.9 Seal the plate. RCF 1000g centrifuge 5 minutes, discard supernatant and add 150 μL Wash Buffer.

2.10 Subject to flow cytometry analysis. If not being analyzed immediately, store at 2~8 °C and avoided

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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.

Instruments tested by this assay were represented in Table 7.

Table 7. 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 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. Set voltages to make sure the PE signal located at right range side of the detection platforms but not with an outside distribution.

3.2.2 APC 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. Set voltages to make sure the APC signal located at right range side of the detection platforms but not with an outside distribution.

3.3 Select medium flow rate for the samples running on the flow cytometry.

3.4 Set up 500 events or beads per 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, and read one by one.*

4.1.3 Resuspend beads by pipetting up and down or thoroughly vortex.

4.1.4 Load the plate and start acquisition.

4.1.5 Confirm each plex based on the APC intensity, record the median fluorescence intensity (MFI) of PE channel.

4.2 Data analysis

4.2.1 Two-log-linear fit curve model is applied 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: *If two-log-linear fit curve model is not suitable for the data analysis, other regression such as four parameters can be another choice.*

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

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【Quick Guide】

Capture Beads

Calibrator or Samples

Biotinylated Detection Ab

SA-PE

1

- Add 30 μL Calibrator or Samples
- Add 100 μL Capture Beads
- Add 100 μL Detection Antibody

Shake, avoid light ↓ 18-25℃ 120 minutes

2

- 1000g centrifuge 5 minutes, discard supernatant and add 200 μL Wash Buffer

↓

3

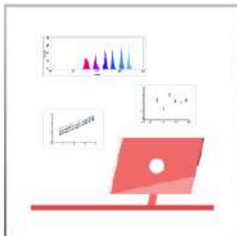
- 1000g centrifuge 5 minutes, discard supernatant and add 200 μL SA-PE Solution

Shake, avoid light ↓ 18-25℃ 30 minutes

4

- 1000g centrifuge 5 minutes, discard supernatant and add 200 μL Wash Buffer
- 1000g centrifuge 5 minutes, discard supernatant and add 150 μL Wash Buffer

↓



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- Subject to flow cytometry analysis

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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 PMT gain or voltage value of PE fluorescent channel is too high.	Adjust PMT gain or voltage of PE fluorescent channel, ensuring 6

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the 6 peaks obtained in APC-count histogram.		bead populations in APC-PE scatter plot.
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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