

Research use only, not for diagnostic use!



# Mouse Multiplex-Analyte Flow Assay Kit

## User Guide



**Attention! Before use, please read the Safety Data Sheet (SDS) and follow the instructions to wear laboratory clothing, protective glasses, and gloves!**



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## Chapter 1: KIT DESCRIPTION

### 【How it Works】

#### 【Introduction】

Soluble Protein detection, particularly for cytokines and chemokines detection are widely used in disease diagnosis and scientific research. The traditional technique for detecting and quantifying proteins one at a time is the enzyme-linked immunosorbent assay (ELISA). However, using this method to detect multiple proteins becomes challenging as the processing time and required sample volume increase. Multiple cytokines can be detected by the flow cytometry at the same time, and multiple results can be obtained in a single sample test, reducing sample volume and saving time. magEasyQplex is magnetic bead-based quantitative assay, using two kinds of fluorescence encoded beads to detect soluble proteins with a common flow cytometer equipped with blue and red lasers. The assay is fully quantitative and has been optimized for serum, plasma, and cell culture supernatant.

magEasyQplex assays are magnetic bead-based immunoassays using the same basic principle as sandwich immunoassays. Beads are differentiated by two internal fluorescence intensities. Each bead set is conjugated with a specific capture antibody on its surface. When a selected panel of capture beads are mixed and incubated with one sample containing target analytes, each analyte will be captured by its specific beads. After washing, a biotinylated detection antibody cocktail is added to form bead-analyte-detection antibody sandwiches. PE-conjugated streptavidin molecules (SA-PE) is added, the intensity of PE fluorescence, in proportion to the amount of bound analytes, was assessed by flow cytometry at wavelength of 575 nm approximately. The intensity of APC and APC-Cy7 fluorescence are applied to classify bead populations at wavelength of 670 nm approximately.

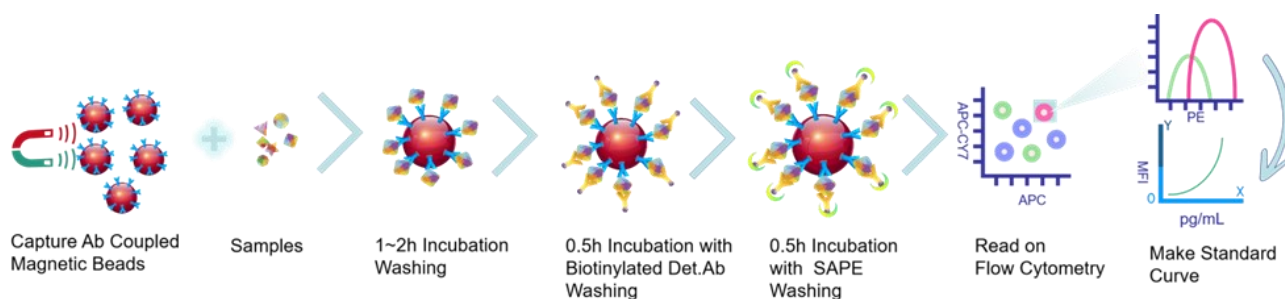


Figure 1: A schematic showing how magnetic bead-based multiplex protein detection with flow cytometry.

#### 【How Beads Differentiated】

Different Panel uses different sets of beads, which depends on the number of analytes to be detected. Each set has the same size and can be identified based on

its unique internal fluorescence intensities. The internal dye can be detected using APC and APC-Cy7 channels. Each analyte is associated with a particular bead set as indicated in COA. Below is an example.

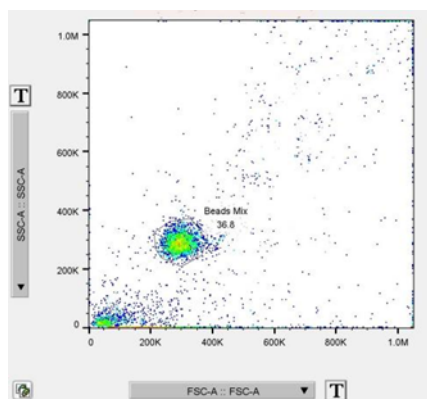


Figure 2. Beads mix identified based on FSC and SSC profiles

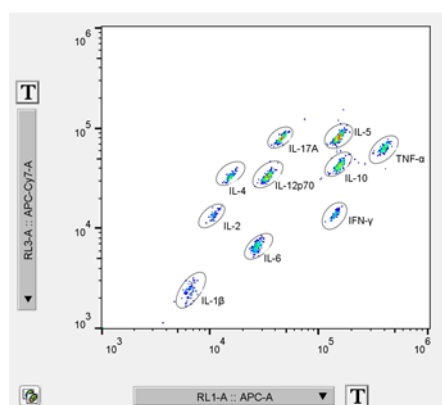


Figure 3. Each bead set differentiated using APC and APC-Cy7 channels

## 【Materials Provided and Storage】

The kit contains reagents at least for 96 tests, Materials provided are listed in the table below.

When assayed in duplicate, it's enough for

an 7-point standard curve and 40 samples. Upon receipt, store the kit at 2°C to 8°C. During long term storage, Lyophilized Protein Standard Mix are suggested to be stored at -20°C. All reagents are stable until the expiration date.

Table 1. Materials Supplied of magEsayQplex Assay Kit

Materials supplied	Basic kit	Single analyte kit	Premixed panels
Lyophilized Protein Standard Mix		✓	✓
Antibody Coupled Magnetic Beads, Single Analyte (50X)		✓	
Antibody Coupled Magnetic Beads, Premixed Panels (1X)			✓
50 X Biotinylated Detection Antibody		✓	✓
1x Assay Buffer	✓		
1x Assay Diluent	✓		
20X Wash Buffer Concentrate	✓		
50X Streptavidin-PE (SA-PE)	✓		
1X Reading Buffer	✓		
PCR 8-Tube Strip	✓		
96-well Flat Bottom Plate	✓		
Plate Seals	✓		

### 【Required Materials not Supplied】

- Orbital 96-well plate shaker (with ability to reach a speed of  $700 \pm 50$  rpm)
- Flow cytometer with blue and red lasers capable of measuring phycoerythrin (PE) , allophycocyanin (APC) and APC-Cy7 channels
- Vortex mixer
- Micro-centrifuge
- Hand-Held Magnetic Plate Washer for 96-well plate with flat bottom ((e.g., Cat. LZMag-96-1)
- Distilled water
- Fresh cell culture medium for running cell culture supernatant samples
- 1.5 ml polypropylene microcentrifuge tubes or similar
- Adjustable single and multichannel pipettes with disposable tips and low-volume reservoirs
- Beakers, flasks, and cylinders necessary for preparation of reagents

## Chapter 2: ASSAY PROTOCOL

### 【Workflow at A Glance】



#### Reconstitute and serial diluent lyophilized protein standards

##### Add beads mix

- Vortex beads 30 sec. Diluent and mix as needed.
- Add 50  $\mu\text{L}$  of 1x beads or beads mix to each well. Remove liquid.
- Add samples and standards
- Add the following according to sample type:
  - ◇ For serum, plasma samples: Add 30 $\mu\text{L}$  of 1x Assay Buffer and 15  $\mu\text{L}$  of samples. Add 45 $\mu\text{L}$  diluted standards to standard wells. For background wells, add 45  $\mu\text{L}$  1x Assay Buffer.
  - ◇ For cell culture supernatant samples: Add 45  $\mu\text{L}$  of standards or samples. For background wells, add 45  $\mu\text{L}$  1x Assay Buffer (or cell culture medium) .
- Seal the plate and incubate with shaking for 60-120 mins at room temperature.
- Remove liquid and wash twice.

##### Add detection antibody

- Diluent and mix detection antibody as needed.
- Add 25  $\mu\text{L}$  of Detection Antibody Mix (1x).
- Seal the plate and incubate with shaking for 30 min at room temperature.
- Remove liquid and wash twice.

##### Add Streptavidin-PE

- Diluent and Add 45  $\mu\text{L}$  of 1x Streptavidin-PE.
- Seal the plate and incubate with shaking for 30 min at room temperature.

- Remove liquid and wash three times

##### Resuspend beads

- Add 150  $\mu\text{L}$  1x Reading Buffer.
- Seal the plate and shake for 5 min at room temperature.



##### Set up and acquire data on Flow cytometer

## 【Assay Protocol】



### Samples Preparation

#### Preparation of Serum Samples:

- Allow the blood to clot for 20-30 minutes and centrifuge for 10-20 minutes at 1,000 x g.
- Collect the serum fraction. Use immediately or store aliquots at  $-80^{\circ}\text{C}$ . Avoid multiple freeze/thaw cycles.
- When using frozen samples, it is recommended that samples are thawed completely, mixed and centrifuged to remove particulates prior to use.

#### Preparation of Plasma Samples:

- Collect samples in sodium citrate or EDTA tubes. If using heparin as an anti-coagulant, no more than 10 IU of heparin per mL of blood collected should be used to prevent assay interference that can result in a false positive signal.
- Centrifuge samples at  $1,000 \times g$  at  $4^{\circ}\text{C}$  for 10 minutes within 30 minutes of collection.
- Collect the plasma fraction. Use immediately or store aliquots at  $-80^{\circ}\text{C}$ .

#### Preparation of cell culture supernatants:

- Centrifuge samples at 1,400 rpm for 10 minutes at  $4^{\circ}\text{C}$  to remove debris.

- Aliquot the clarified medium into clean polypropylene micro-centrifuge tubes.
- Use immediately or store aliquots at  $-80^{\circ}\text{C}$ . Avoid multiple freeze/thaw cycles.



### Reagent Preparation

#### Prepare 1x Wash Buffer

- Bring the Wash Buffer Concentrate (20x) to room temperature and vortex for 15 seconds. Mix 20 mL of the Wash Buffer Concentrate (20x) with 980 mL ddH<sub>2</sub>O. Mix gently to avoid foaming. Wash Buffer (1x) can be stored at  $2-8^{\circ}\text{C}$  for up to 1 week.

#### Prepare 1x Single Bead Mixture

**Note:** Single-analyte kits are provided with concentrated 50x beads, which will require dilution before use. Premixed panels are provided with 1x beads mix and can be used directly.

- Vortex each single bead vial (50x) for 30 seconds, then add 100  $\mu\text{L}$  of each 50x single bead to a mixing bottle if using a whole plate (otherwise adjust the volume accordingly).
- Add 1x Wash Buffer to a final volume of 5 mL. To mix 2 or more different single bead set, follow table 2 below (using a whole plate):

Table 2: Dilution and Combination of Single Bead Sets

Number of different single bead set to be mixed	Total volume of mixed bead solution	Volume of 1x Wash Buffer to add
1	100 $\mu\text{L}$	4900 $\mu\text{L}$
2	200 $\mu\text{L}$	4800 $\mu\text{L}$
3	300 $\mu\text{L}$	4700 $\mu\text{L}$
4	400 $\mu\text{L}$	4600 $\mu\text{L}$
5	500 $\mu\text{L}$	4500 $\mu\text{L}$
6	600 $\mu\text{L}$	4400 $\mu\text{L}$

### Prepare 1x detection antibody mixture

- Centrifuge the biotinylated detection antibody vials for 30 seconds at 1000x g prior to removing the cap.
- Add 60  $\mu$ L of each 50x detection anti-

body to the mixing bottle and bring volume to a total of 3 mL using assay diluent for the whole plate usage.

- To mix 2 or more different detection antibody, follow table 3 below.

Table 3: Dilution and Combination of Different Biotinylated Detection Antibody

Number of vials of detection antibody to be mixed	Total volume of detection antibody	Volume of diluent to add
1	60 $\mu$ L	2940 $\mu$ L
2	120 $\mu$ L	2880 $\mu$ L
3	180 $\mu$ L	2820 $\mu$ L
4	240 $\mu$ L	2760 $\mu$ L
5	300 $\mu$ L	2700 $\mu$ L
6	360 $\mu$ L	2640 $\mu$ L

### Prepare 1x Streptavidin-PE

- Centrifuge the 50x Streptavidin-PE vials for 30 seconds at 1000x g prior to removing the cap.
- Add 90  $\mu$ L of 50x Streptavidin-PE to the mixing bottle and bring volume to a total of 4.5 mL using assay diluent for the whole plate usage. Otherwise adjust the volume accordingly.

Dissolve the powder thoroughly and gently by pipetting up and down for 5-10 times to avoid foaming. Incubate on ice for 10 minutes to ensure complete reconstitution.

- ◇ For >1 bottle of standards provided: Add 100 $\mu$ L 1x Assay Buffer to each bottle. Dissolve the powder thoroughly and gently by pipetting up and down for 5-10 times to avoid foaming. Incubate on ice for 10 minutes to ensure complete reconstitution.

- Pool each stock bottle into one final 400 $\mu$ L standard mix, as below table:



### Standards Preparation

#### Reconstitute the Lyophilized Protein Standard Mix

- ◇ For only 1 bottle of standard provided: Add 400 $\mu$ L 1x Assay Buffer to the bottle.

Table 4: Combination of Different Standard Bottles

Number of bottles of standards to be mixed	Reconstitution volume per bottle	Volume of diluent to add	Total Volume of Standard 1
2	100 $\mu$ L	200 $\mu$ L	400 $\mu$ L
3	100 $\mu$ L	100 $\mu$ L	400 $\mu$ L
4	100 $\mu$ L	0 $\mu$ L	400 $\mu$ L

**Note: Use the Lyophilized Protein Standard Mix within 1 hour of preparation.**



### Prepare 3-fold serial dilution

- Label the tubes in the 8-Tube Strip: Std1, Std2, Std3, Std4, Std5, Std6, Std7 and Blank.
- Add 120  $\mu\text{L}$  1x Assay Buffer as applicable to tube Std2, Std3, Std4, Std5, Std6, Std7 and Blank.
- Pipette 180  $\mu\text{L}$  reconstitution standard mix to tube Std1, and transfer 60  $\mu\text{L}$  into tube Std2 and mix gently. Perform more serial dilutions by adding 60  $\mu\text{L}$  of Std2 to tube Std3, mix, and so on. (See figure below)

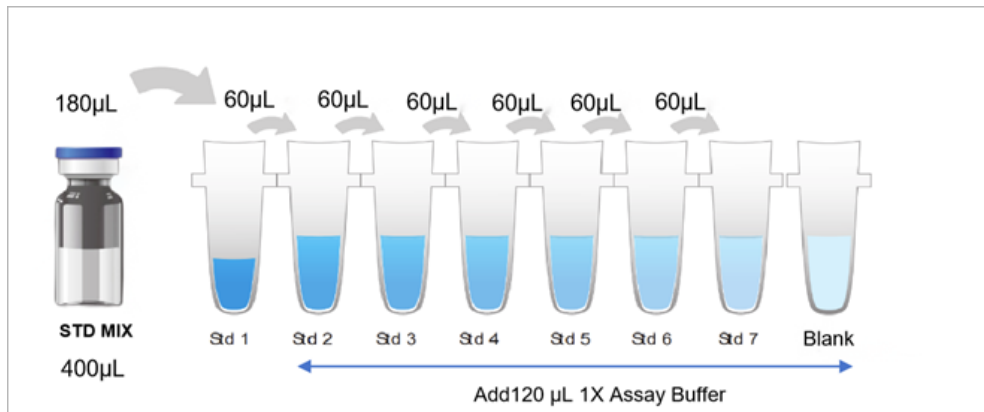


Figure 4. 3-fold serial dilution



### Assay Procedure

- Define the plate map by marking the standard, sample, and blank wells using the plate map in Appendix A, "Recommended plate layout".
- Vortex the 1x Bead Mix vial for 30 seconds at high speed and add 50  $\mu\text{L}$  to each well of the plate.
- Place the plate on the Hand-Held Magnetic Plate Washer and wait 2 minutes to allow the beads to settle on the bottom of each well. Remove the liquid by quickly inverting the washer/plate assembly over a waste container. Using paper towels to remove any residual liquid. Add 150  $\mu\text{L}$  1x Wash Buffer into each well and wait 60 seconds. Remove the liquid, then remove the plate from the Magnetic Plate Washer.
- Add samples and standards to the plate.
  - ◇ **For cell culture supernatants:** add 45  $\mu\text{L}$  prepared standards, blank and samples to the designated wells.
  - ◇ **For serum/plasma,** add 30  $\mu\text{L}$  1x Assay Buffer and 15  $\mu\text{L}$  samples to each sample well. Add 45  $\mu\text{L}$  prepared standards and blank to the designated wells.
- Seal the plate with a plate sealer and shake at 700 $\pm$ 50 rpm for 60-120 minutes at room temperature.
  - Note: This step can also be performed overnight at 4°C.**
- Shake the 96-well plate for 30 minutes at room temperature at 700 $\pm$ 50rpm, then transfer the plate to 4°C and store on a level surface. After overnight incubation, shake the plate for an additional 30 minutes at 700 $\pm$ 50 rpm at room temperature.
- Remove and discard the Plate Seal. Place the plate on the Hand-Held Magnetic Plate Washer and wait 2 minutes before remove the liquid. Add 150  $\mu\text{L}$  of 1x Wash Buffer into each well and wait

30 seconds before remove the liquid.  
Wash 2 time totally.

- Add 25  $\mu$ L 1x detection antibody solution to each well. Gently tap the plate to evenly distribute the solution in the wells. Seal the plate and shake at 600 rpm for 30 minutes at room temperature.
- Wash the plate twice following step 6.
- Add 45  $\mu$ L of SA-PE solution to each well. Seal the plate and shake at  $700\pm 50$  rpm for 30 minutes at room temperature.
- Wash the plate three times following step 6.
- Add 150  $\mu$ L 1x Reading Buffer into each well and shake the plate at  $700\pm 50$  rpm for 5 minutes at room temperature.
- Prepare samples for analysis on a flow cytometer: Load plate to automated high throughput sampler or transfer samples to compatible tubes.

## Chapter 3: FLOW CYTOMETER SETUP AND DATA ACQUISITION

- Perform standard Quality Control steps to prepare the flow cytometer, then set up the flow cytometer as you would for normal use. Compensation may be needed depends on the configuration of flow cytometer.
- Start a new experiment with FSC, SSC, PE, APC, APC-Cy7 channels.
- Create the following plots :
  - ◇ Create a dot plot with FSC/SSC in linear display mode.
  - ◇ Create a FSC-A/FSC-H (linear mode) daughter population for “Single beads” to limit doublets or higher complexes.
  - ◇ Create a new dot plot from “Single beads” parent gate. Gate “Each bead set

”populations based on APC (log mode) and APC-Cy7 (log mode).

- ◇ Create a histograms of PE (log mode) from the “Each bead set ”parent populations.
- ◇ Both Areas and Heights should be collected.
- Run part of the Blank Beads prepared in blank well of the plate to set channel voltage.
- Adjust FS and SS gains so that the bead populations are on scale and create Gate “Beads Mix” (Figure 5).
- Adjust APC and APC-Cy7 PMT voltage so that each bead set are on scale (Figure 6)
- Adjust PE PMT voltage so that the PE mean fluorescent intensity (MFI) is around  $10^2$  for each bead population.

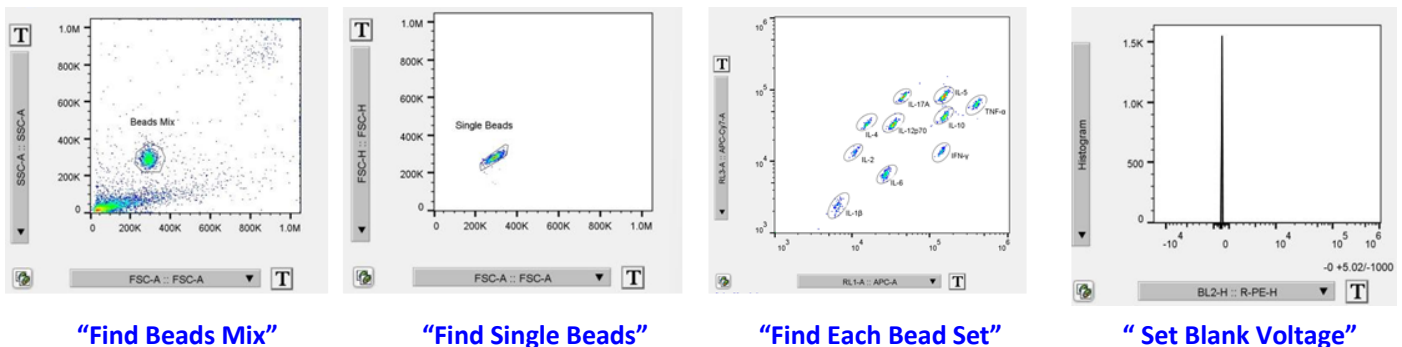


Fig 5. Procedure of create Gate “Beads Mix”

- Run part of the S1 Beads prepared in S1 well of the plate to verify PE channel voltage.
- ◇ Verify all the bead populations on the PE-axis are on scale and PE MFI of S1 is around  $10^5$ .
- ◇ Adjust PE PMT voltage if needed. If adjustment is needed, make sure re-run the Blank.
- Set the number of each bead set to be acquired to at least 100 per target population for best results. More beads will improve data accuracy.
- Following setup, run all standards, blanks and samples.
- Create a statistical view to show the PE MFI for each bead set. The MFI of all analytes can be exported in Excel format. Researchers can also export raw data as “FCS” files and analyze in FlowJo software.

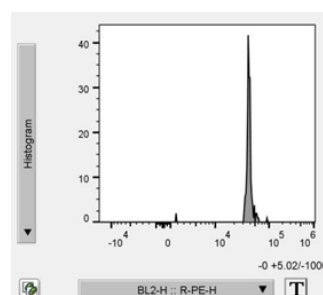


Fig 6. Set S1 Voltage

## Chapter 4: DATA ANALYSIS

- Generate the best standard curve of each target. 5P logistic (5PL) or 4PL fit is recommended. The parameter of the x-axis is concentration (in pg/ml), the parameter of the y-axis is given as MFI of standards/samples minus MFI of blank. Take 10 targets as example:

- The concentration of each target per sample is calculated based on each standard curve. If sample have been diluted, the concentration read from the standard curve must be multiplied by the dilution factor. A standard curve must be run with each assay since the MFI values will vary from experiment to experiment.

Table 5: Acquired MFIs correspond to concentration

	IFN- $\gamma$		IL-10		IL-12p70		IL-17A		IL-1 $\beta$	
	MFI	Conc.(pg/mL)	MFI	Conc.(pg/mL)	MFI	Conc.(pg/mL)	MFI	Conc.(pg/mL)	MFI	Conc.(pg/mL)
Blank	72.3	0.0	72.3	0.0	79.5	0.0	72.3	0.0	72.3	0.0
S1	15254.0	6000.0	20211.0	10000.0	8168.0	10000.0	21739.0	6000.0	11955.0	10000.0
S2	11483.0	2000.0	12892.0	3333.3	5054.0	3333.3	17166.0	2000.0	5655.0	3333.3
S3	5513.0	666.7	6523.0	1111.1	2237.0	1111.1	10059.0	666.7	2221.0	1111.1
S4	1765.0	222.2	2707.0	370.4	783.0	370.4	4306.0	222.2	732.0	370.4
S5	579.0	74.1	1046.0	123.5	275.0	123.5	1585.0	74.1	246.0	123.5
S6	246.0	24.7	398.0	41.2	145.0	41.2	594.0	24.7	116.0	41.2
S7	101.0	8.2	166.0	13.7	94.0	13.7	224.0	8.2	86.8	13.7
	IL-2		IL-4		IL-5		IL-6		TNF-alpha	
	MFI	Conc.(pg/mL)	MFI	Conc.(pg/mL)	MFI	Conc.(pg/mL)	MFI	Conc.(pg/mL)	MFI	Conc.(pg/mL)
Blank	72.3	0.0	72.3	0.0	72.3	0.0	72.3	0.0	79.5	0.0
S1	15378.0	10000.0	19009.0	10000.0	20472.0	10000.0	10444.0	10000.0	12669.0	6000.0
S2	10929.0	3333.3	15011.0	3333.3	13646.0	3333.3	5567.0	3333.3	7599.0	2000.0
S3	5460.0	1111.1	8763.0	1111.1	6002.0	1111.1	2260.0	1111.1	3194.0	666.7
S4	1931.0	370.4	3933.0	370.4	1870.0	370.4	695.0	370.4	1104.0	222.2
S5	514.0	123.5	1503.0	123.5	601.0	123.5	217.0	123.5	369.0	74.1
S6	217.0	41.2	550.0	41.2	239.0	41.2	108.0	41.2	166.0	24.7
S7	101.0	13.7	203.0	13.7	116.0	13.7	79.5	13.7	101.0	8.2

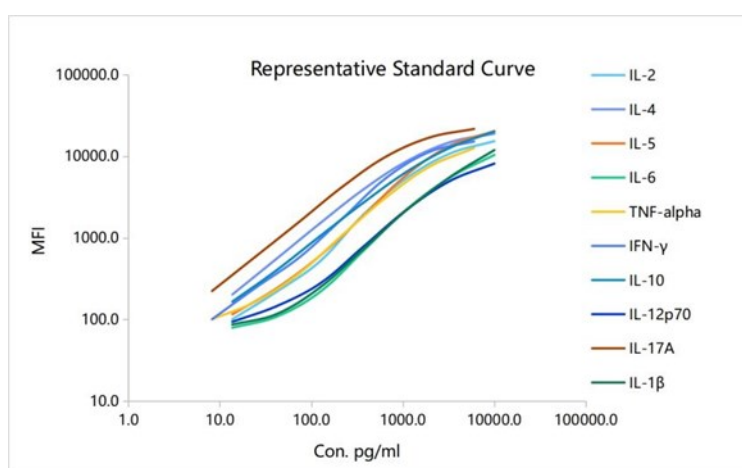


Fig 7. Representative Standard Curve

## Chapter 5: TROUBLESHOOTING

Issue	Possible Cause	Solution
Poor standard curve	Lyophilized standard mix not properly prepared	Serially Lyophilized standard mix according to manual.
	Lyophilized standard mix degraded or not properly diluted	Reconstitute the Lyophilized standard mix on ice before making serial dilutions.
	Cross-contamination from neighboring wells	Avoid overflowing wells during wash steps.
Low standard curve signal	Wrong or short incubation time	Incubate according to manual
	PMT value for FL2/PE set too low	Ensure the PMT setting for the reporter channel is appropriate
	Lyophilized standard mix not properly prepared or diluted	Reconstitute the standard mix and do serial dilutions according to manual.
Low bead counts	Bead sets are not mixed properly	Fully vortex beads before mix and transfer to plate
	Beads missed during washing	Make sure to wait for 2 mins when the plate firstly is put on the Hand-Held Magnetic Plate Washer before removing liquid.
	Sample may cause some beads to aggregate.	Centrifuge samples. If high lipid content is present, remove lipid layer after centrifuge. Sample may need dilution if too viscous.
	Probe might be clogged.	Sonicate sample probe
High background	Cross-contamination from neighboring wells	Avoid overflowing wells during wash steps.
	Insufficient washes	Increase number of washes after SAPE incubation
	PMT value for PE set too high	Ensure the PMT setting of PE is appropriate

APPENDIX: RECOMMENDED PLATE LAYOUT

	1	2	3	4	5	6	7	8	9	10	11	12
A	S1	S1										
B	S2	S2										
C	S3	S3										
D	S4	S4										
E	S5	S5										
F	S6	S6										
G	S7	S7										
H	Blank	Blank										



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