RayPlex® Custom Human Multiplex Bead Array Kit

Quantitative measurement of proteins in liquid phase by flow cytometry

User Manual
Last revised June 25, 2020

Catalog numbers:
FAH-CUST-100 (100 tests)
FAH-CUST-500 (500 tests)

RayBiotech
Empowering your proteomics

ISO 13485 Certified

3607 Parkway Lane, Suite 200
Peachtree Corners, GA 30092
Tel: 1-888-494-8555 (toll-free) or 770-729-2992; Fax:770-206-2393
Web: www.raybiotech.com Email: info@raybiotech.com

Table of Contents

I. Introduction

RayBiotech is an industry leader in protein and antibody array technologies and services. The RayPlex Multiplex Cytometric Bead Arrays are designed to detect a wide range of common cytokines, serological proteins, intracellular signal molecules and biomarkers for a wide variety of disease states including cancer, inflammation, cardiovascular disease, and others. The RayPlex array panels are easily customized from the vast sandwich antibody pair library used in our popular Quantibody® array. Because RayBiotech's ELISA kits use these same antibodies, corresponding validation assays are readily available for follow-up studies.

The RayPlex antibody-coated bead technology significantly reduces the amount of sample required compared to standard ELISA, while delivering unparalleled flexibility in analyte combination when designing a customized panel. RayPlex arrays are compatible with most common flow cytometers.

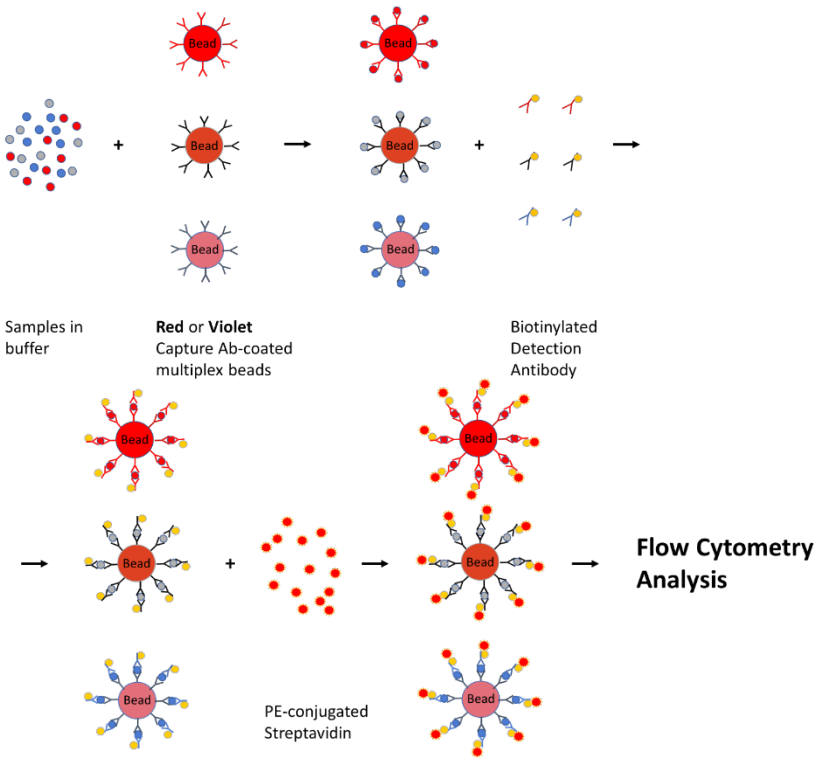
II. Overview

A 12-plex kit is shown as an example.

RayPlex Multiplex Bead Antibody Array – Custom Human Kit	CXCL13, FCRL3, CCL21, CD23, CD137, C1R, Slamf6, IgM, CD22, TNFRSF17, Siglec6, TFNRSF4
Format	Flow Cytometry-based. This kit can be performed by a flow cytometer with blue laser (PE channel) and red laser (APC channel)
Detection Method	Flow Cytometry
Minimal Sample Volume	Serum or plasma:12.5 µl; Culture supernatant or cell lysate: 25 µl
Assay Duration	4 hours

III. How it Works

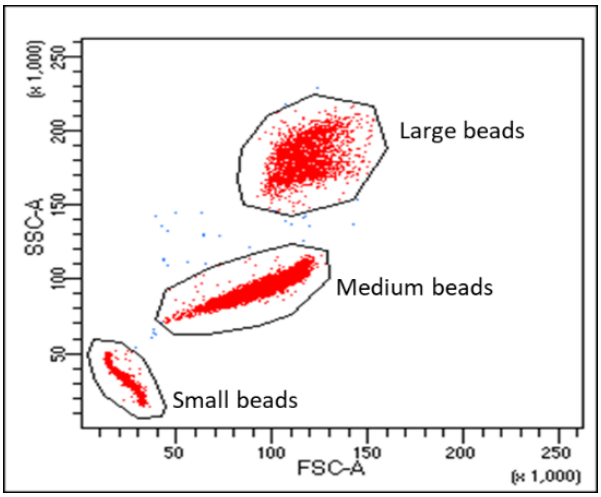
RayBio® Multiplex Bead Antibody Array is a sandwich-based assay. The general assay procedures are outlined as follows.



IV. Bead ID and Distribution

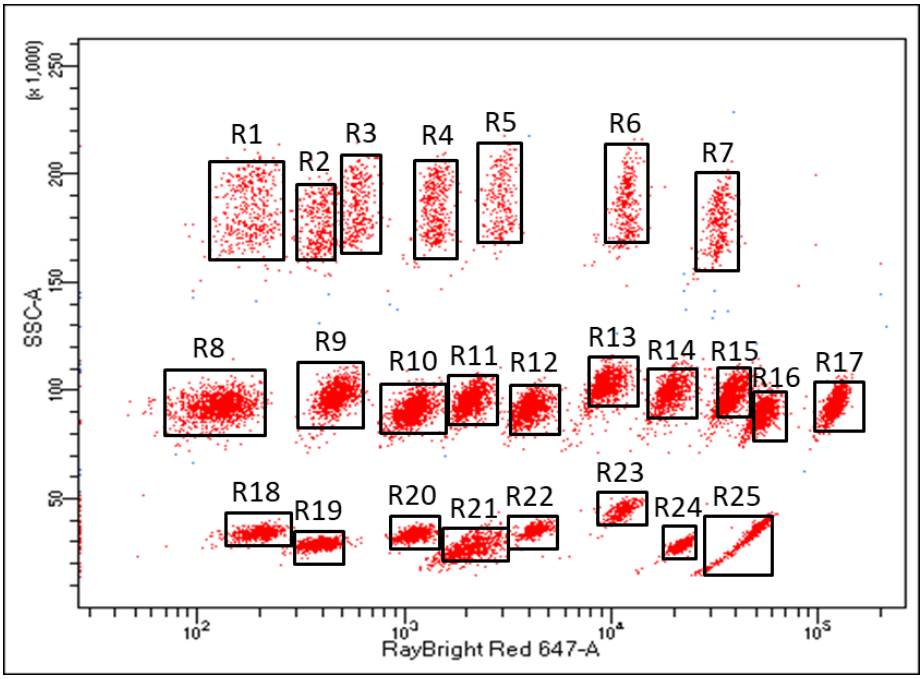
RayBio® Multiplex Beads consist of three sizes: large, medium and small (Figure 1 and 2). Target-specific beads ID is shown as follows.

Figure 1. Multiplex beads size



Bead ID R1 - R7: Large Size Beads
Bead ID R8 - R17: Medium Size Beads
Bead ID R18 - R25: Small Size Beads

Figure 2. Multiplex beads by size and color intensity distribution



Specific Panel Bead ID Distribution

FHA-CUST-100 (500)	
Bead ID	**Targets**
R1	CXCL13
R2	FCRL3
R3	
R4	CCL21
R5	CD22
R6	CD23
R7	CD137
R8	C1R
R9	Slamf6
R10	IgM
R11	
R12	TNFRSF17
R13	Siglec6
R14	TNFRSF4
R15	
R16	
R17	
R18	
R19	
R20	
R21	
R22	
R23	
R24	
R25	

V. Materials Provided

Upon receipt, all components of the RayPlex Multiplex Cytometric Bead Array kit should be stored at 4˚C. Lyophilized protein standard should be stored at -80˚C.

If stored in this manner, kit will retain complete activity for at least 6 months.

Item	Description	100 Tests	500 Tests
1	RayPlex Custom Human Multiplex Bead Cocktail	2.5 ml	12.5 ml
2	5X Assay Diluent	20 ml	100 ml
3	20X Wash Buffer	10 ml	50 ml
4	V-shaped 96-well Microplate	1	5
5	Lyophilized Protein Standard Mix	1	5
6	Detection Antibody Cocktail (biotinylated)	500 µl	2.5 ml
7	Streptavidin-PE	50 µl	250 µl
8	Manual	1	1

Additional Materials Required

- Orbital 96-well plate shaker (with ability to reach 1000 rpm)
- Flow cytometer with violet, blue and red lasers
- Aluminum foil
- Distilled water
- 1.5ml polypropylene microcentrifuge tubes or similar
- Microcentrifuge
- Rainbow calibration particles
- Optional: High throughput sampler (HTS) for 96-well plate reading

VI. Assay Protocol

A. Preparation of Samples

- We recommend the following parameters for your samples:
  25 µl of original or diluted serum, plasma, cell culture media, or other body fluid.
- If testing serum and plasma, dilute samples with Assay Diluent at least 1:1
  - **Note:** Levels of target protein(s) may vary between different samples. Optimal dilution factors for each sample must be determined empirically by the investigator.
- If serum-containing conditioned media is required, it is highly recommended that complete medium be used as a control since many types of sera contains assay targets, or cross-reactive proteins.

- It's recommended to use a 96-well round bottom plate (mirror plate) to prepare samples and then transfer those samples to the test plate or test tubes.

  If you experience high background or the readings exceed the detection range, further dilution of your sample is recommended.

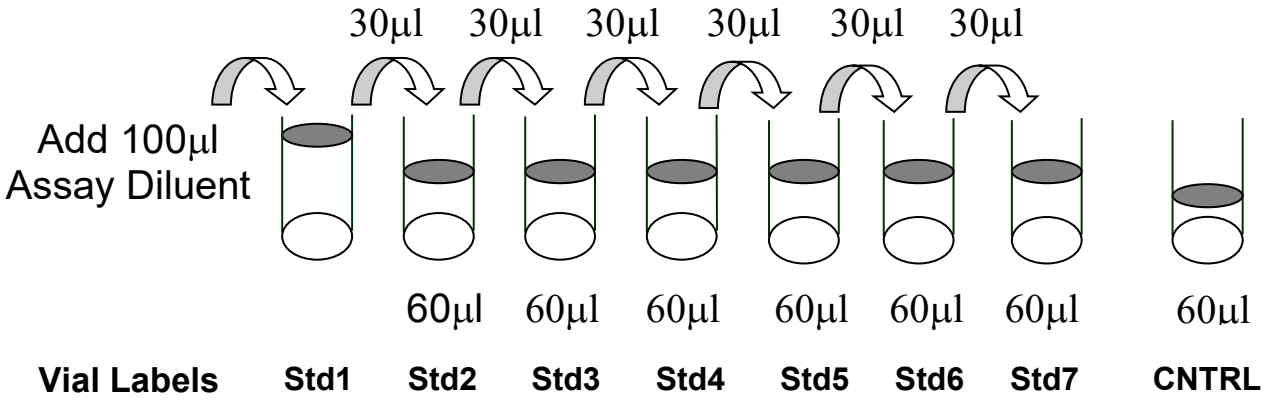
B. Preparation of Reagents

- Keep all reagents on ice.
- Multiplex beads cocktail must be vortexed for 30 seconds each time before use. Use 25 µl beads/test.
- Dilute 5x Assay Diluent with DI H_2O to create 1x Assay Diluent.
- Detection antibody cocktail should be diluted 1:10 in Assay Diluent as working stock. Dilute only what is needed to perform the tests for each experiment. Use 50 µl/test.
- Streptavidin-PE is diluted 1:100 in Assay Diluent as working stock. Dilute only what is needed to perform the tests for each experiment. Use 50 µl/test.
- Protect fluorescent multiplex beads from frequent exposure to light.
- Dilute 20x wash buffer with DI H_2O to create 1x wash buffer. Dilute only what is needed to perform the tests for each experiment.

C. Preparation of Protein Standards

Note: Reconstitute the lyophilized standard within one hour of usage.

Prepare serial dilutions of protein standards



1. Reconstitute the Protein Standard Mix (lyophilized) by adding 100 μl Assay Diluent to the tube. For best recovery, always quick-spin vial prior to opening. Dissolve the powder thoroughly by gentle mixing, and label this tube as Std1.
2. Label 6 clean microcentrifuge tubes as Std2 to Std7. Add 60 μl Assay Diluent to each of the tubes.
3. Pipette 30 μl Std1 into tube Std2 and mix gently. Perform 5 more serial dilutions by adding 30 μl Std2 to tube Std3, mix, and so on.
4. Add 60 μl Assay Diluent to another tube labeled as CNTRL. Do not add standard or samples to the CNTRL tube. This tube will be used as the negative control.

Note: Since the starting concentration of each protein standard may be different, the concentrations from Std1 to Std7 for each protein are varied. The exact concentrations can be found in section VII.

D. Assay Procedures

1. Prepare a V-shaped 96-well microplate and mark positions for standard and samples. Duplicate tests are recommended for all standards and samples for best data quality.
2. Add 25 µl of RayPlex Multiplex Bead cocktail to the corresponding wells to be used.
3. Add 25 µl standard or pre-diluted sample to the above corresponding wells. Total volume is each well is 50 µl. Place plate on an orbital plate shaker. Shake at 1000 rpm at room temperature for 2 hours.

   Note: This step may be done overnight at 4˚C for higher signal results. Longer incubation time is preferable for higher signal or for lesser protein concentrations.
4. Wash the beads by adding 200 µl wash buffer and spin down at 1000 g for 5 minutes at room temperature, remove the supernatant using a multichannel pipet. Repeat this wash step one more time.

5. Add 50 µl of biotinylated detection antibody cocktail (pre-titrated, see Section B, Preparation of Reagents) to each well. Resuspend the beads by gently pipetting and incubate on an orbital shaker at 1000 rpm, room temperature for 1 hour.

6. Wash plate once as outlined in step 4. Add 50 µl of Streptavidin-PE (pre-titrated, see Section B, Preparation of Reagents) to each well, incubate on an orbital shaker at 1000 rpm, room temperature for 30 minutes.

7. Wash plate once as outlined in step 4. Resuspend in 200 µl of Assay Diluent and run the samples on a flow cytometer with an HTS, or transfer samples to standard FACS tubes for manually reading.

E. Flow Cytometer Set-up and Data Acquisition

NOTE: RayBiotech currently offers two kinds of multiplex beads. Our "Red Beads" (emission in the APC channel, and the same as RayBright® 647) while our "Blue Beads" (emission in the Violet 450nm channel). If only using Red Beads, a flow cytometer with Blue and Red lasers is required. If using both Violet and Red Beads, a flow cytometer equipped with 3-lasers (violet, blue and red) is required. Perform standard QC and Optimization for the cytometer during setup, then set up the cytometer as you would for normal use. Overall, compensation is not necessary when only using Red Beads and Streptavidin-PE. If beads are too intense in the APC channel, there might be a smiling effect (curved population grouping), in which case manually adjust PE and APC compensation to correct it.

1. Depending on the brand of the flow cytometer, you may need to start the acquisition software and run QC beads before proceeding further.

2. Start a new experiment with PE and APC channels.

3. Adjust voltage for FSC (forward scatter, linear mode) and SSC (side scatter, linear mode) so that the major bead populations are shown (Figure 1). Create FSC-H/FSC-A daughter population for "Single beads" selection to remove and limit doublets or higher complexes (Figure 3A).

4. Create a new dot plot from "Single beads" parent gate, and gate on Large, Medium, and/or Small beads (Figure 3B).

5. Create new dot plot from the Large, Medium, and/or or Small beads parent gate. Gate populations for all bead populations for the assay based on SSC (linear mode) and APC (log mode). Adjust APC channel PMT voltage so that all populations are evenly distributed throughout in a prominent area (Figure 3C).

6. Create APC by PE (using log scale for both) dot plot from the Large, Medium, and/or Small beads parent populations. Run a small amount of the negative beads sample and the top standard beads as a sample. Adjust PE voltage so that in negative beads for each population the PE MFI is around 10^1 - 10^2.

7. Following setup, run standard and samples (as in Figure 3D).

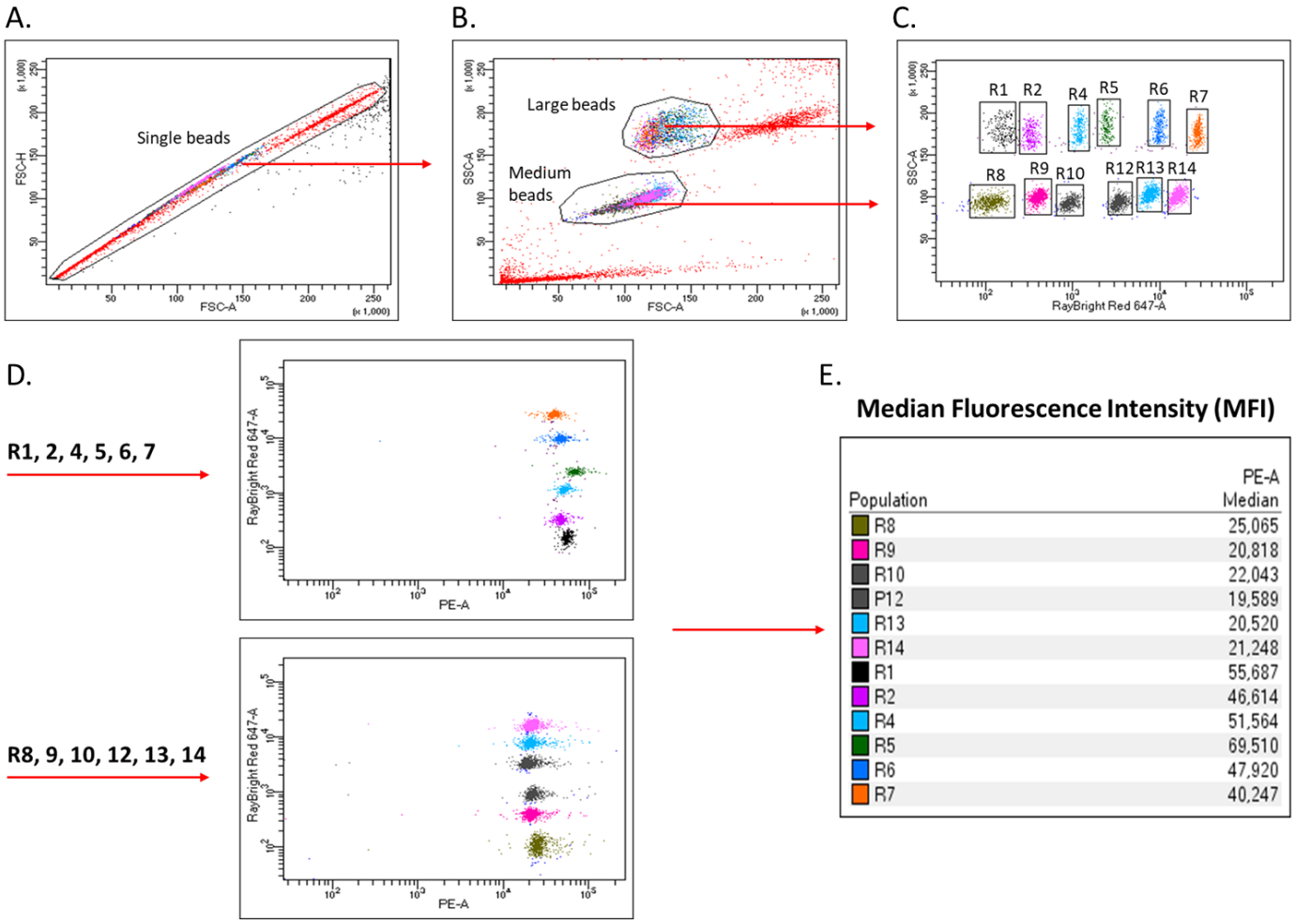
8. Create a statistics view to show MFI for each population (Figure 3E). **MFI of all analytes for all samples can be exported in Excel format by "Batch Analysis" of a whole specimen in FACSDiva.**

9. To keep the testing consistent upon each assay, use of rainbow calibration particles (rainbow beads, mid-range preferred) can allow the standardization of the assay if run in each assay before collecting samples.

10. An HTS is preferred as the 96-well microplate can be used directly. If an HTS is not available, transfer sample to FACS tubes for acquisition. Set the number of bead events to be acquired to at least 300 per target population for best results. More beads will equate to improve accuracy.

    Note: MFI data of each analytes for all samples can also be acquired by exporting the entire complete dataset as an FCS file. Analyze data in FlowJo or equivalent software and create and export the PE MFI for all populations of all standards and samples in Excel/similar format.

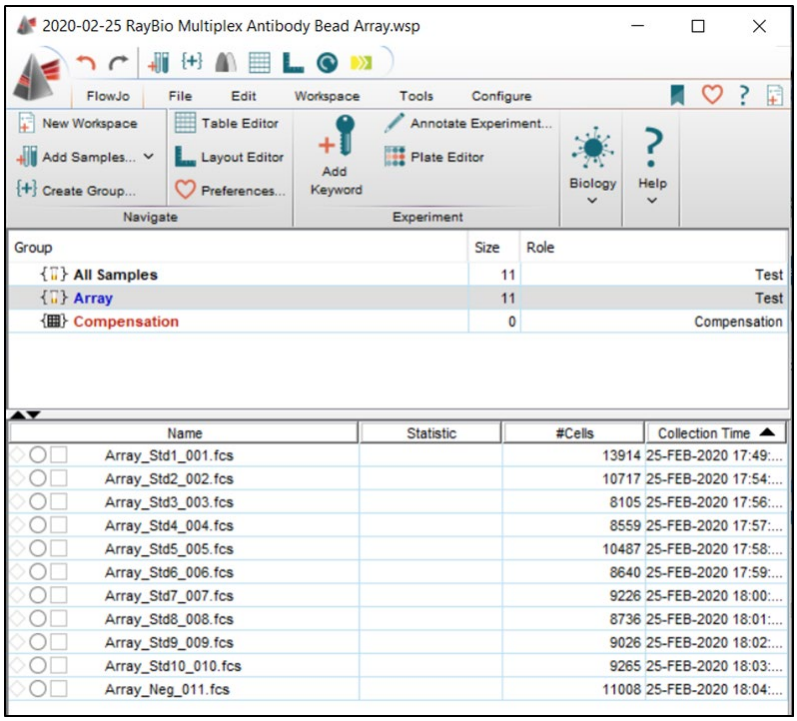
Figure 3. A Representative Image of Flow Cytometer Set-up for a Test Using Large and Medium Red Beads.



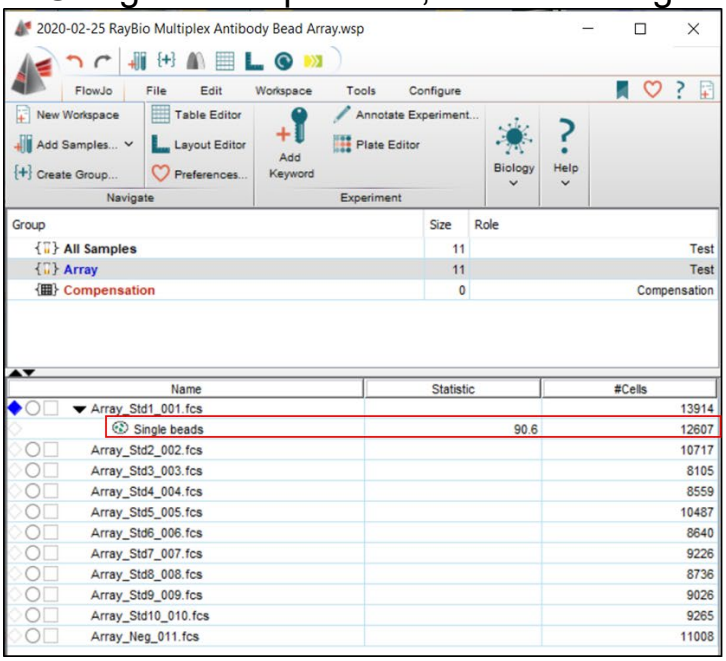
F. Data Analysis using FlowJo

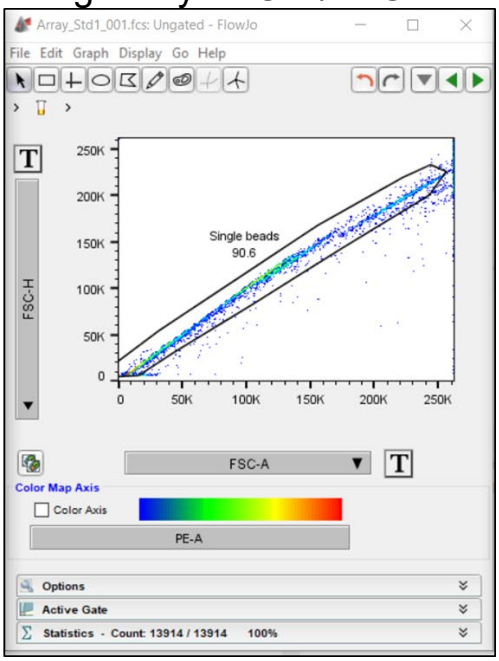
The following bead array data set analysis is based on FlowJo software. If you require assistance in analyzing your data, please contact us info@raybiotech.com

1. Open FlowJo and drag FCS files or a folder contains FCS files to a new workspace. An example “Array” folder is used for this instruction. Save the analysis as a WSP file with a new name.

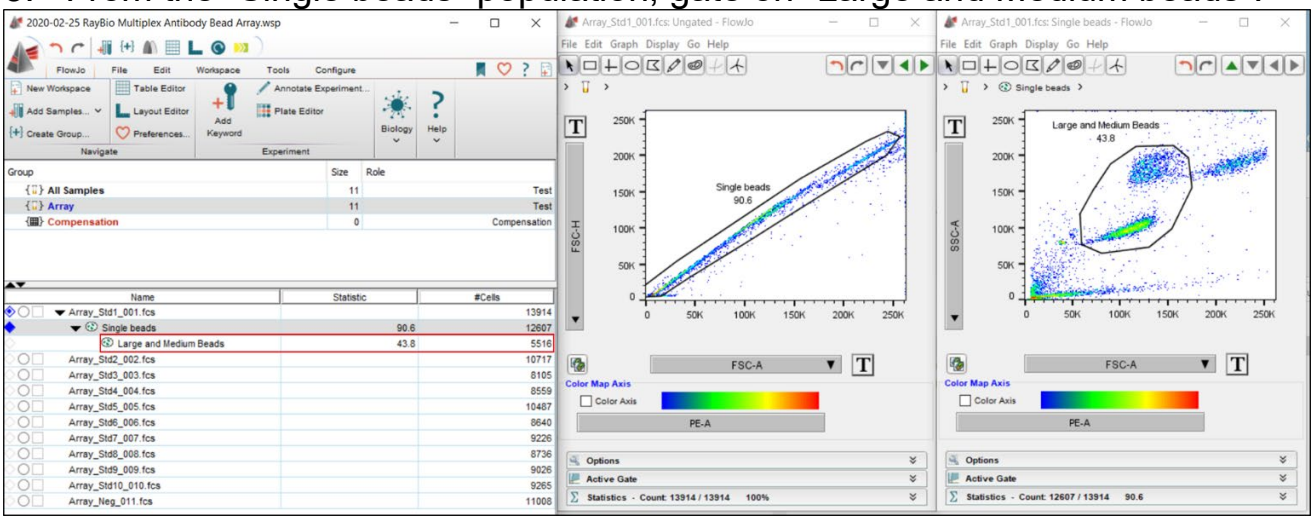


2. Using the sample Std1, create a “Single beads” gate by FSC-H/FSC-A.

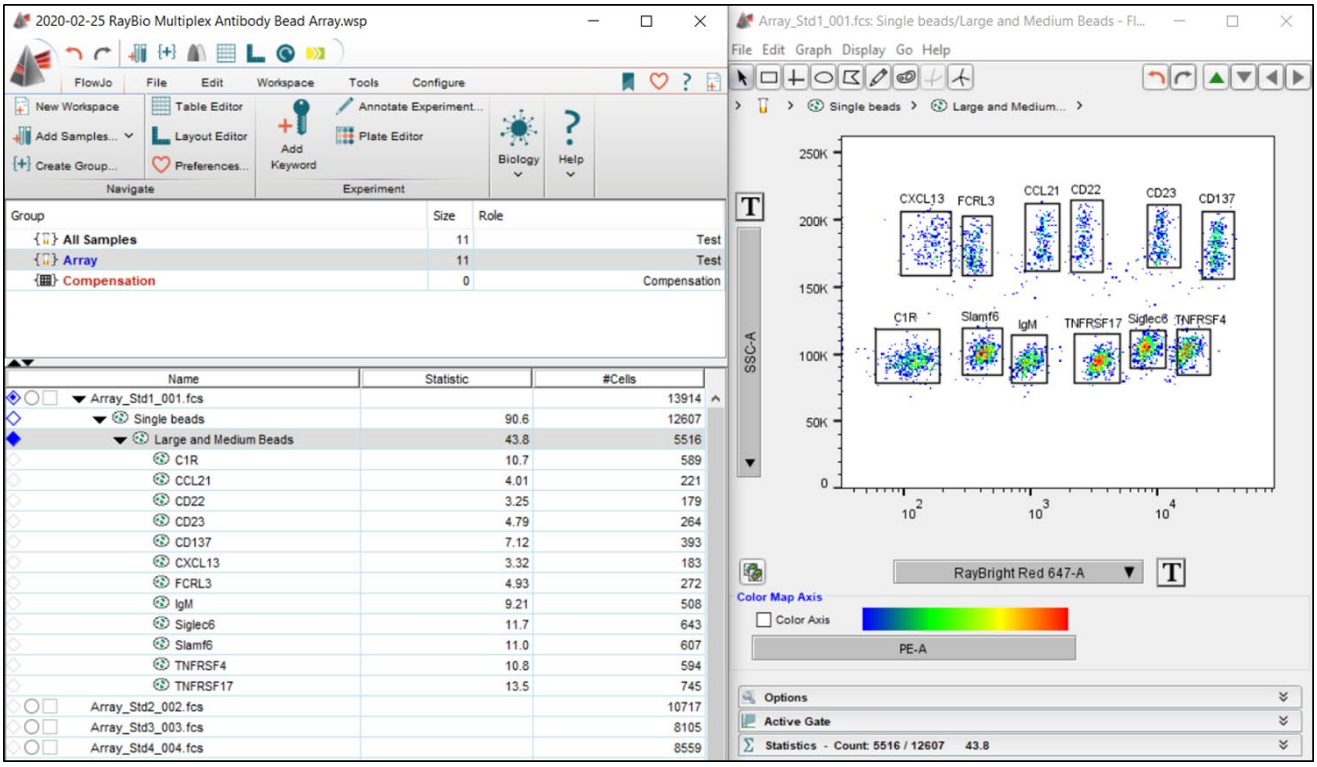




3. From the “Single beads” population, gate on “Large and Medium beads”.

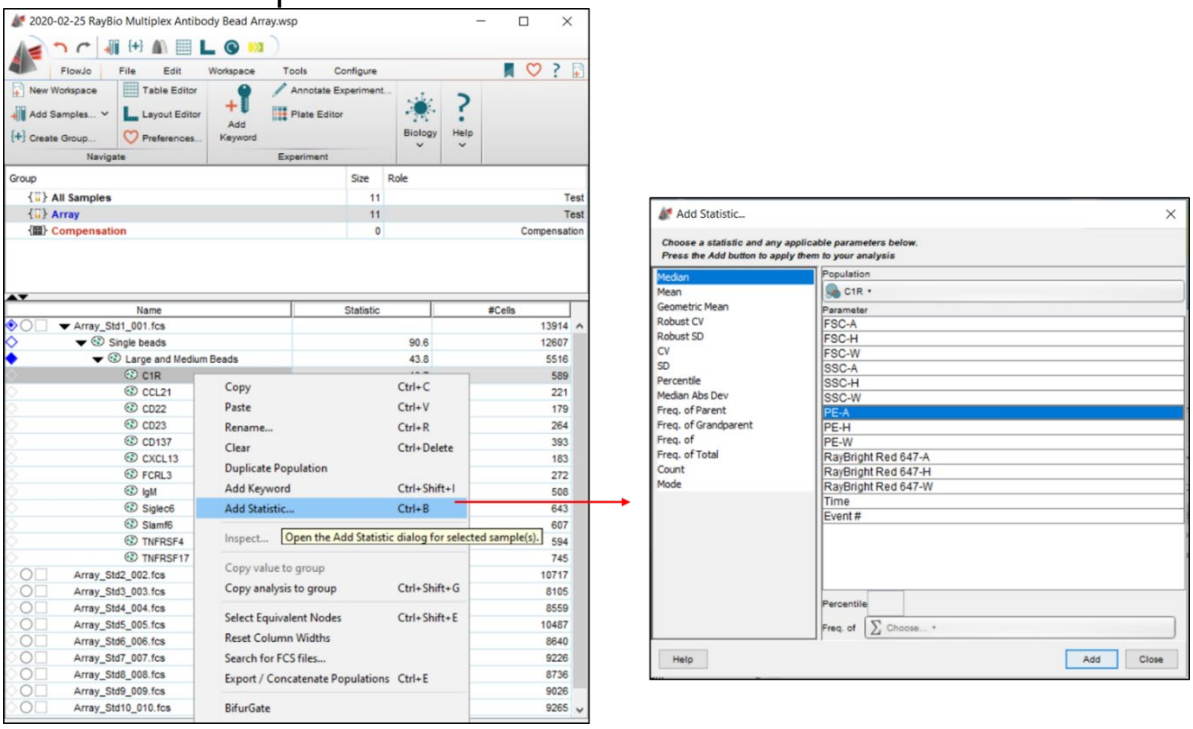


4. Within the “Large and Medium beads” parent gate, create each target bead populations using SSC(Linear) by APC(Log). Adjust axis settings to allow separation of targets populations.



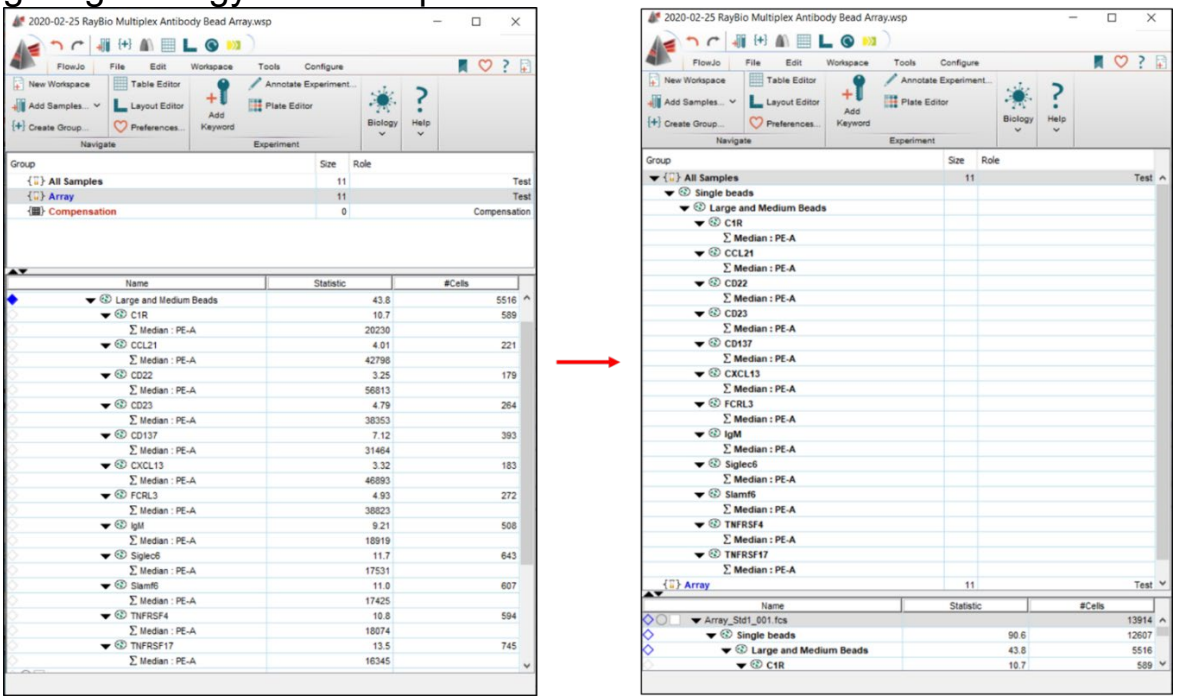
Name	Statistic	#Cells
Array_Std1_001.fcs		13914
Single beads	90.6	12607
Large and Medium Beads	43.8	5516
C1R	10.7	589
CCL21	4.01	221
CD22	3.25	179
CD23	4.79	264
CD137	7.12	393
CXCL13	3.32	183
FCRL3	4.93	272
IgM	9.21	508
Siglec6	11.7	643
Slamf6	11.0	607
TNFRSF4	10.8	594
TNFRSF17	13.5	745
Array_Std2_002.fcs		10717
Array_Std3_003.fcs		8105
Array_Std4_004.fcs		8559

5. For each population, add the “Median” (MFI) statistics for the PE-A channel as shown in the picture.

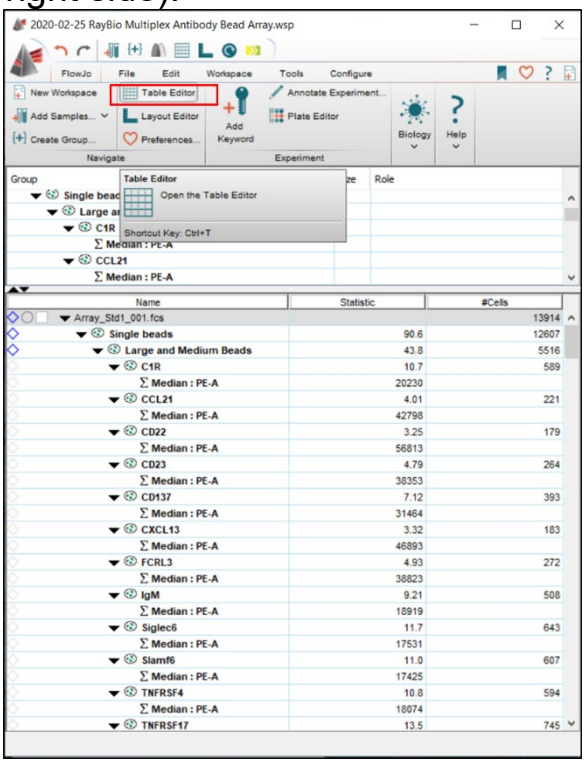


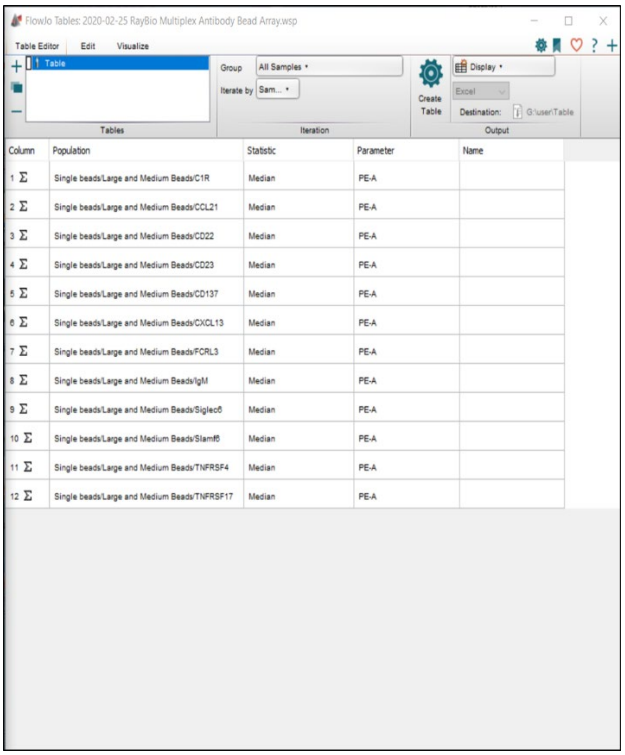
6. Add the “Median” (MFI of PE-A) population to all target groups.

7. Copy all gates from Std1 to the “All samples” group at the top to apply this gating strategy to all samples.

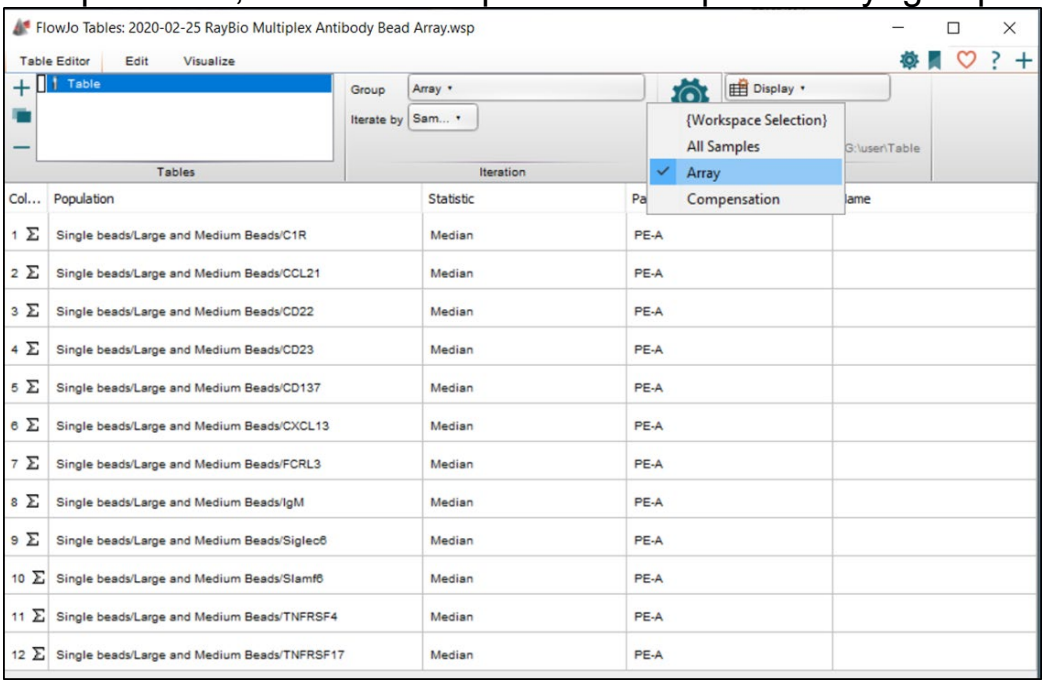


8. Click “Table Editor” as shown in the red frame to open the table. Drag the icon indicating the Median of each population to the table (see the table on right side).

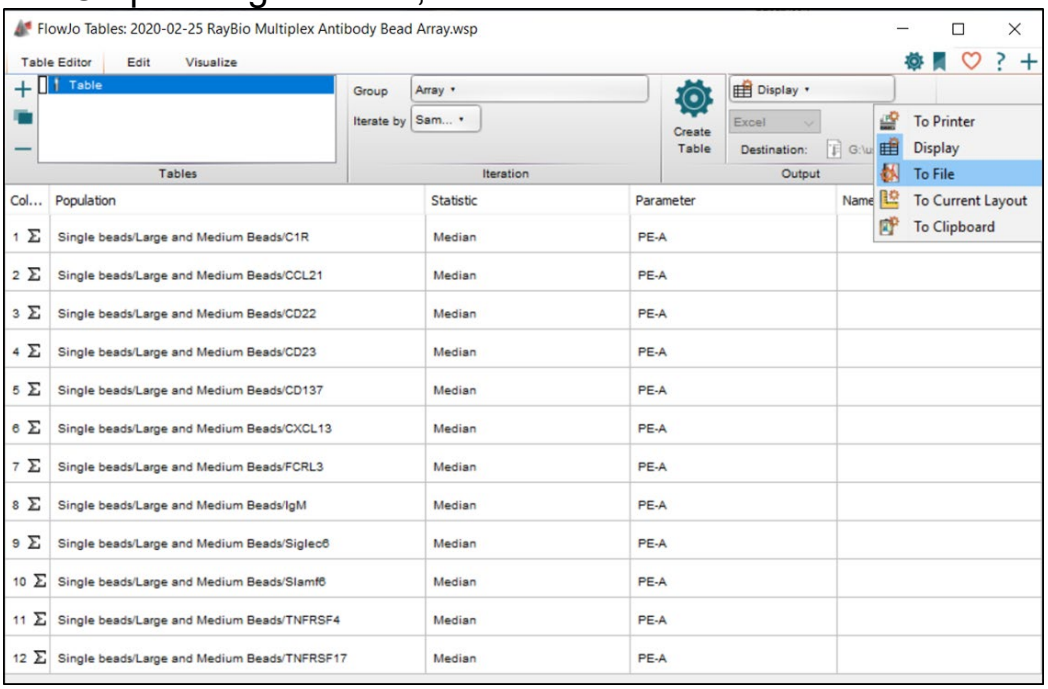




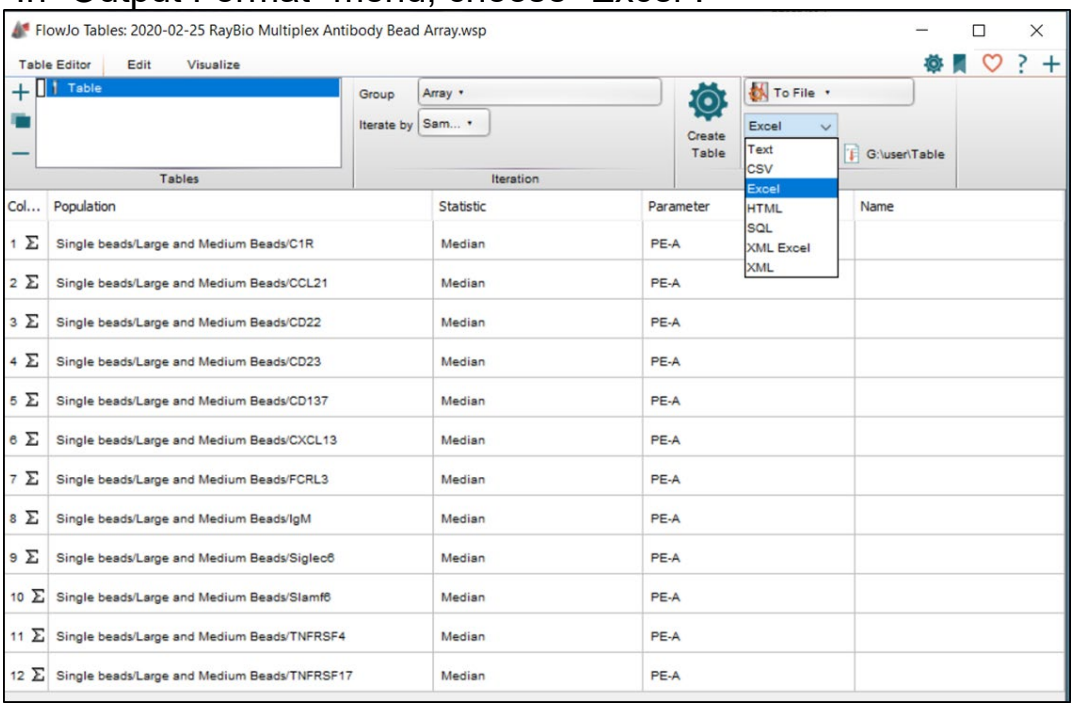
9. At top of table, choose “Group” menu and pick “Array” group.



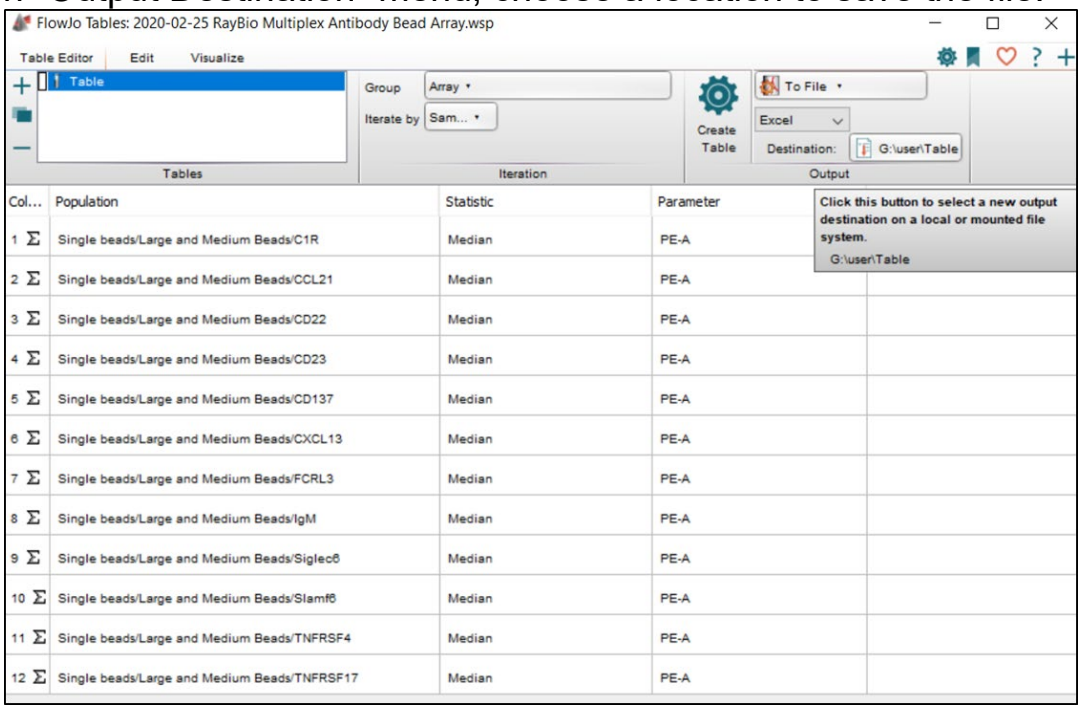
10. In “Output Target” menu, choose “To file”.



11. In “Output Format” menu, choose “Excel”.



12. In “Output Destination” menu, choose a location to save the file.



13. Click “Create Table” icon to export Excel table.

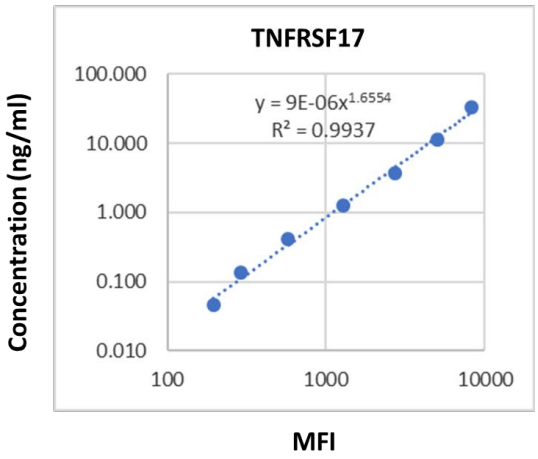
Median Fluorescence Intensity (MFI) of PE-A

	C1R	CCL21	CD22	CD23	CD137	CXCL13	FCRL3	IgM	Siglec6	Slamf6	TNFRSF4	TNFRSF17
Array_Std1_001.fcs	20230	42798	56813	38353	31464	46893	38823	18919	17531	17425	18074	16345
Array_Std2_002.fcs	10040	19985	29157	17425	13368	21831	18129	9475	8674	8112	9475	8236
Array_Std3_003.fcs	6358	11445	17214	10477	7656	13287	10477	5838	5264	4952	5750	5059
Array_Std4_004.fcs	3754	6243	9979	5646	3893	7941	5838	3184	2897	2636	3014	2743
Array_Std5_005.fcs	2035	3005	5697	2810	2143	4520	2734	1538	1412	1289	1469	1292
Array_Std6_006.fcs	925	1289	3395	1312	1047	2853	1209	671	626	570	661	580
Array_Std7_007.fcs	444	607	2230	636	561	1920	563	342	311	279	345	293
Array_Std8_008.fcs	267	436	1644	449	417	1501	401	218	197	177	234	196
Array_Std9_009.fcs	205	353	1835	370	346	965	306	174	167	145	198	158
Array_Std10_010.fcs	181	324	1649	360	330	587	300	162	155	134	187	146
Array_Neg_011.fcs	135	244	1575	266	254	234	209	108	100	82.6	128	95.9

14. In Excel, create a standard curve (log-log mode preferred) for each analyte based on MFI and calculate values for all analytes of each sample. A representative analysis is shown for one of the analytes.

TNFRSF17

	MFI	Std (ng/ml)
Std 1	16345	100.000
Std 2	8236	33.333
Std 3	5059	11.111
Std 4	2743	3.704
Std 5	1292	1.235
Std 6	580	0.412
Std 7	293	0.137
Std 8	196	0.046
Std 9	158	0.015
Std 10	146	0.005
Neg	96	0.00



15. Calculate the concentration of each analyte of each sample based on the regression curve formula, or a different formula of your choice (linear, multiparameter, etc). It's recommended that researchers create a template in Excel, so that further assays can be easily analyzed in subsequent experiments. for the same assay in the future.

VII. 8-Point Standard Protein Concentrations

After reconstitution of the lyophilized protein standard mix, the 8-point protein concentration used for generating the standard curve of a given antigen is listed below.

Concentration of standards

Antigen	Negative	Std7	Std6	Std5	Std4	Std3	Std2	Std1	Unit
CXCL13	0.00	0.14	0.41	1.23	3.70	11.11	33.33	100.00	ng/mL
FCRL3	0.00	0.14	0.41	1.23	3.70	11.11	33.33	100.00	ng/mL
CCL21	0.00	0.14	0.41	1.23	3.70	11.11	33.33	100.00	ng/mL
CD23	0.00	0.14	0.41	1.23	3.70	11.11	33.33	100.00	ng/mL
CD137	0.00	0.14	0.41	1.23	3.70	11.11	33.33	100.00	ng/mL
C1R	0.00	0.14	0.41	1.23	3.70	11.11	33.33	100.00	ng/mL
Slamf6	0.00	0.14	0.41	1.23	3.70	11.11	33.33	100.00	ng/mL
IgM	0.00	0.14	0.41	1.23	3.70	11.11	33.33	100.00	ng/mL
CD22	0.00	0.14	0.41	1.23	3.70	11.11	33.33	100.00	ng/mL
TNFRSF17	0.00	0.14	0.41	1.23	3.70	11.11	33.33	100.00	ng/mL
Siglec6	0.00	0.14	0.41	1.23	3.70	11.11	33.33	100.00	ng/mL
TNFRSF4	0.00	0.14	0.41	1.23	3.70	11.11	33.33	100.00	ng/mL

VIII. Troubleshooting Guide

Problem	Cause	Recommendation
Weak Signal	Inadequate detection	Increase sample and beads incubation time.
	Detection antibody over diluted	Increase detection antibody concentration.
	Too low protein concentration in sample	Don't over dilute samples.
	Too low protein concentration in sample	Don't make too low dilution or concentrate sample.
	Improper storage of kit	Store kit as suggested temperature.
	Reagent evaporation	Cover the incubation plate with adhesive film during incubation.
Poor standard curve	Cross-contamination from neighboring wells	Avoid overflowing wash buffer.
	Too much detection antibody	Optimize the detection antibody
	Standard protein degraded or not property diluted	Reconstitute the lyophilized standard well on ice before making serial dilutions.
High background	Improper flow cytometer setup and optimization	Run QC-beads before assay. Make sure high end signal not out of linear range.
	Insufficient wash	Increase wash time and use more wash buffer.
	Too much detection antibody	Optimize the detection antibody.

IX. Note:

RayBio® is the trademark of RayBiotech Life, Inc.

This product is intended for research only and is not to be used for clinical diagnosis. Our produces may not be resold, modified for resale, or used to manufacture commercial products without written approval by RayBiotech Life, Inc.

Under no circumstances shall RayBiotech be liable for any damages arising out of the use of the materials.

Products are guaranteed for three months from the date of purchase when handled and stored properly. In the event of any defect in quality or merchantability, RayBiotech's liability to buyer for any claim relating to products shall be limited to replacement or refund of the purchase price.

This product is for research use only.