

# RayPlex<sup>®</sup> Mouse Chemokines Array Kit 1

Quantitative Measurement of 12 Mouse Proteins  
by Flow Cytometry

Catalog numbers:  
**FAM-CHEM-1-48 (48 tests)**  
**FAM-CHEM-1-96 (96 tests)**  
**FAM-CHEM-1-192 (192 tests)**  
**FAM-CHEM-1-480 (480 tests)**

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## I. Overview

|                               |   |
|-------------------------------|---|
| <b>Species</b>                | Mouse   |
| <b>Proteins Detected (12)</b> | CCL2 (MCP-1), CXCL11 (I-TAC), CCL4 (MIP-1 $\beta$ ), CX3CL1 (Fractalkine), CCL17 (TARC), CCL20 (MIP-3 $\alpha$ ), CCL22 (MDC), CXCL1 (KC), CCL11 (Eotaxin-1), CXCL9 (MIG), CCL5 (RANTES), CXCL13 (BLC). |
| <b>Format</b>                 | Bead-based  |
| <b>Detection Method</b>       | Flow Cytometry using a flow cytometer equipped with a blue laser (PE channel) and red laser (APC channel)   |
| <b>Minimal Sample Volume</b>  | 25 $\mu$ l per test after dilution. <i>The optimal dilution must be determined empirically by the researcher.</i>   |
| <b>Number of Replicates</b>   | At least 2 replicates. Each replicate is considered a test.   |
| <b>Reproducibility</b>        | See Section VII (page 21) for inter-CV and intra-CV percentages for each protein.   |
| <b>Assay Duration</b>         | 4 hours   |

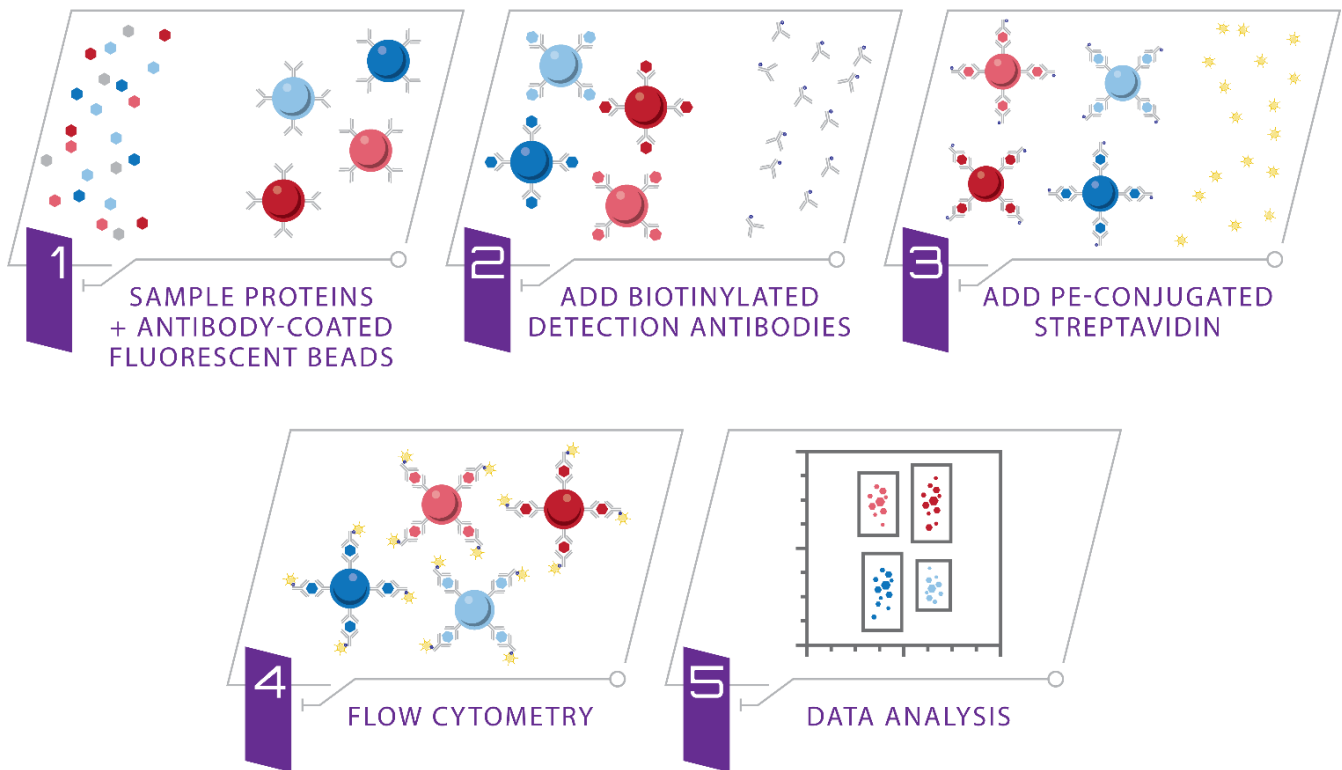
## II. Introduction

Chemokines are involved in many processes including immune surveillance, organ development, angiogenesis, and immune response. Thus, the quantification of chemokine expression is important to better understand disease states, especially those involved in inflammation and immune disorders.

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 – particularly for precious samples with small quantities – becomes challenging as the processing time and required sample volume increase. RayPlex is a multiplexed sandwich- and bead-based quantitative antibody array for the simultaneous detection of multiple proteins using small sample volumes. It combines the versatility of RayBiotech's vast antibody pair library with familiar, reliable flow cytometry methodology. Together this creates a multiplex bead-based array requiring no dedicated instrumentation, only a common flow cytometer equipped with blue and red lasers. The RayPlex Mouse Chemokine Array Kit 1 (12-plex) is a bead-based multiplex assay panel, using fluorescence-encoded beads to detect the above cytokines with a flow cytometer. This panel covers 12 key targets and has been optimized for serum, plasma, and cell culture supernatant.

### III. How it Works

RayPlex arrays are first prepared by immobilizing capture antibodies onto small beads of different sizes and different fluorochromes; there is only one target's capture antibody per bead size-fluorochrome combination. The capture antibodies bind to their specific protein targets during sample incubation, and unbound proteins are removed with washing. Biotinylated detection antibodies and PE-conjugated streptavidin molecules are added, thus enabling protein detection via the PE- streptavidin-biotin-antibody complex. Individual proteins are identified by their specific bead-fluorochrome combination, while the level of PE fluorescence reflects the amount of protein that has been captured onto the beads. The protein amount can be determined (i.e., quantified) by comparing the PE signal to a standard curve generated from purified protein standards at known concentrations.



**A schematic showing how RayPlex enables multiplex protein detection with flow cytometry.**

## IV. Bead ID and Distribution

### 1. General View of RayPlex Multiplex Beads.

As many as four different sizes of beads are used with RayPlex arrays: small, medium-1, medium-2, and large, (Figures 1 and 2). This RayPlex Mouse Chemokines Array Kit 1 uses **medium-1** and **large** beads.

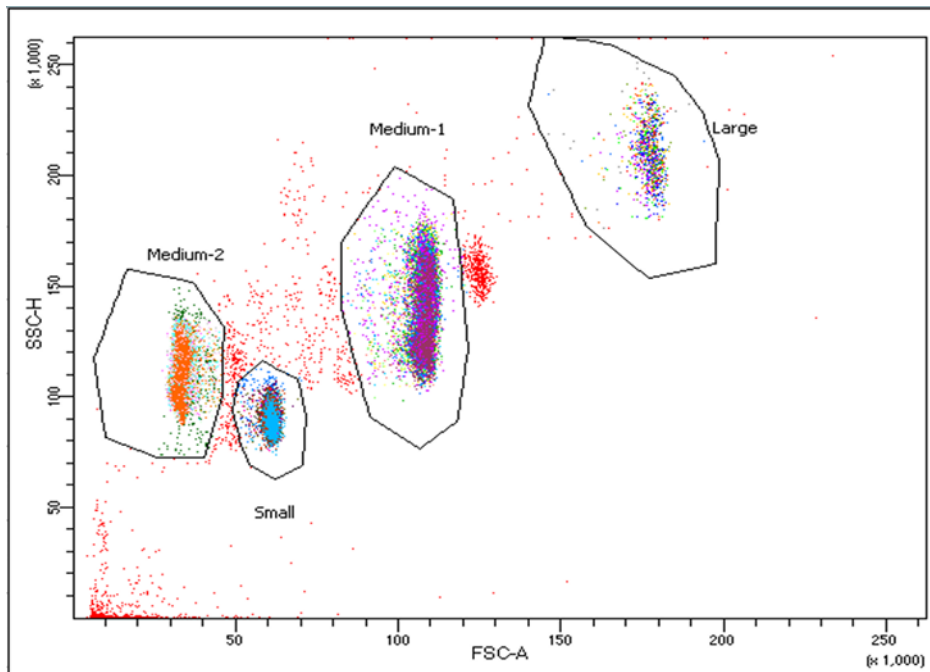
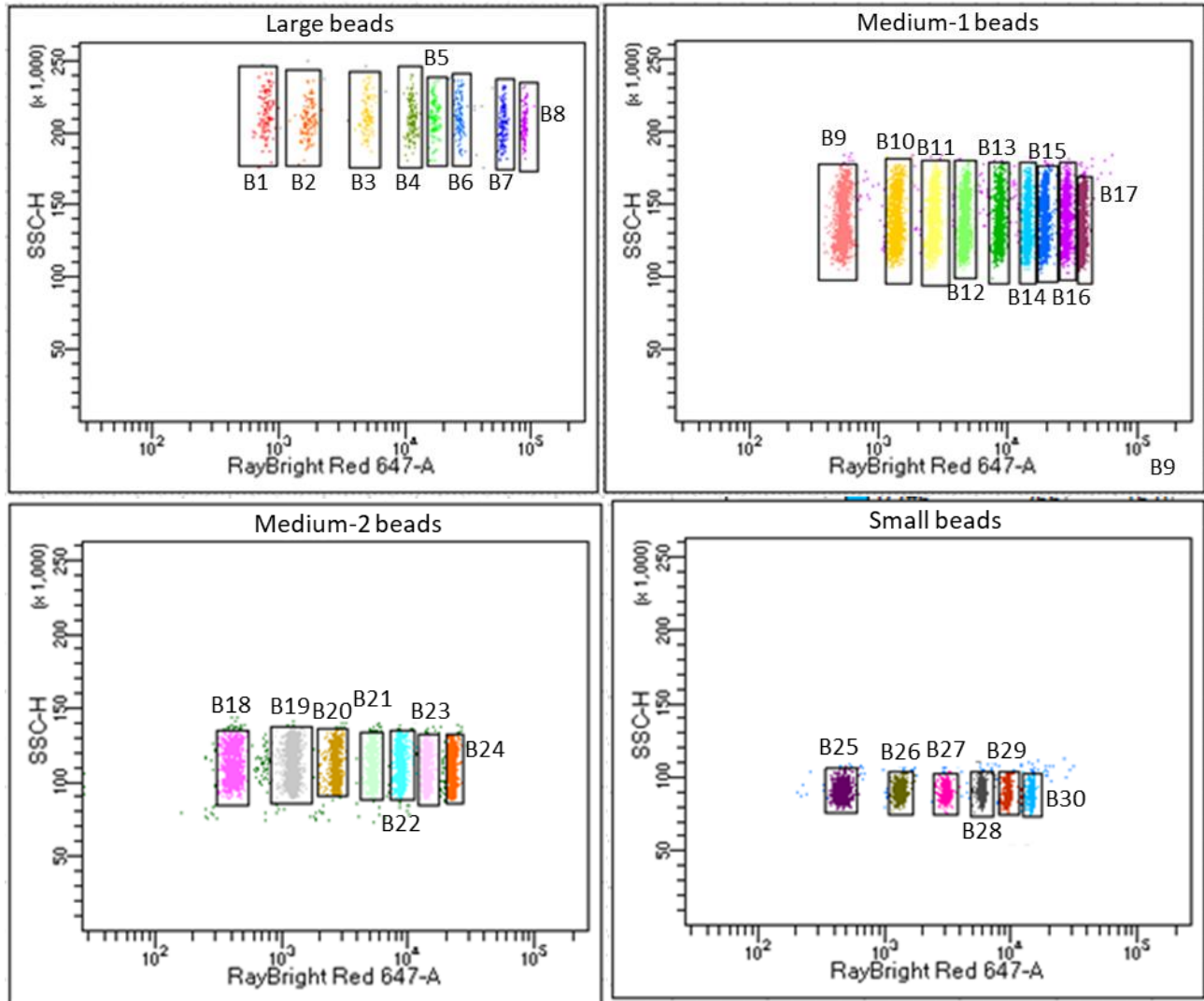


Figure 1. RayPlex uses different size beads

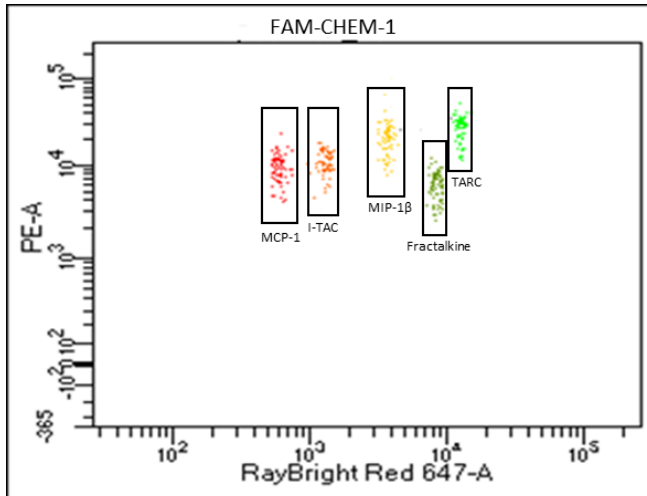


**Figure 2. Different populations of bead size and color combinations (“B#”) enable multiplex protein detection with RayPlex arrays. *Bead ID* B1 – B8: Large Beads. *Bead ID* B9 - B17: Medium-1 Beads. *Bead ID* B18 – B24: Medium-2 Beads. *Bead ID* B25 – B30: Small Beads.**

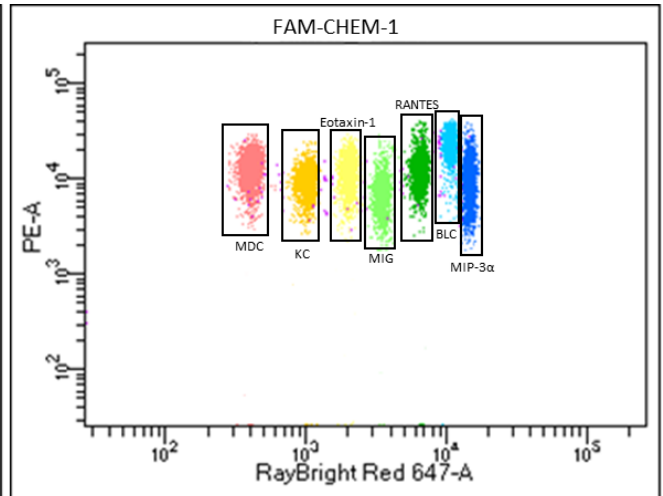
## 2. Target Proteins and associated Bead ID in this kit.

| Bead ID                 | Target |
|-------------------------|--------|
| CCL2 (MCP-1)            | B1     |
| CXCL11 (I-TAC)          | B2     |
| CCL4 (MIP-1 $\beta$ )   | B3     |
| CX3CL1 (Fractalkine)    | B4     |
| CCL17 (TARC)            | B5     |
| CCL22 (MDC)             | B9     |
| CXCL1 (KC)              | B10    |
| CCL11 (Eotaxin-1)       | B11    |
| CXCL9 (MIG)             | B12    |
| CCL5 (RANTES)           | B13    |
| CXCL13 (BLC)            | B14    |
| CCL20 (MIP-3 $\alpha$ ) | B15    |

Large beads



Medium-1 beads





## V. Materials Provided

Upon receipt, the Lyophilized Protein Standard Mix (Item 5) should be stored at -20°C or below. Other components should be stored at 4°C. *Use reagents immediately after preparation.* Additional vials of the Lyophilized Protein Standard Mix (Item 5) can be purchased separately (cat # FAM-CHEM-1-Item 5). If stored at the proper temperature, the kit will retain complete activity for at least 6 months.

| Catalog #        | Description                                | 48 Tests         | Per 96 Tests      |
|------------------|--|------------------|-------------------|
| FAM-CHEM-1-Item1 | RayPlex Multiplex Bead Cocktail            | 1.25 ml x 1 vial | 1.25 ml x 2 vials |
| FAX-AD-1-Item2   | 1X Assay Diluent                           | 25 ml x 1 vial   | 25 ml x 2 vials   |
| FAX-WB-Item3     | 20X Wash Buffer                            | 5 ml x 1 vial    | 5 ml x 2 vials    |
| FAM-SD-1-Item4   | RayBio® Serum Diluent                      | 2.5 ml x 1 vial  | 2.5 ml x 2 vials  |
| FAM-CHEM-1-Item5 | Lyophilized Protein Standard Mix           | 1                | 2                 |
| FAM-CHEM-1-Item6 | 1X Biotinylated Detection AntibodyCocktail | 1.25 ml x 1 vial | 1.25 ml x 2 vials |
| FAX-PE-Item7     | 10X Streptavidin-PE                        | 250 µl x 1 vial  | 250 µl x 2 vials  |
| FAM-CHEM-1-Item8 | Flow Cytometer Setup Bead Cocktail**       | 100 µl x 1 vial  | 100 µl x 1 vial   |
| FAX-VP-Item9     | V-bottom 96-well Microplate                | 1                | 1                 |
| FAX-FP-Item10    | Filter 96-well Microplate                  | 1                | 1                 |
| N/A              | Manual                                     | 1                | 1                 |

### Notes:

- \*\* Flow Cytometer Setup Bead Cocktail (Item 8) is an additional smaller aliquot of Item 1 provided to assist in the setup of the flow cytometer.
- RayBio® Serum Diluent (Item 4) is a pool of normal Mouse serum from several donors with the proteins-of-interest depleted. Different lots may result in batch effects; thus it is recommended that the researcher take this into consideration when planning their experiments. Contact [info@raybiotech.com](mailto:info@raybiotech.com) if a large lot of RayBio® Serum Diluent is required.
- RayBiotech recommends using a Filter 96-well Microplate (Item 10) in lieu of the V-bottom 96-well microplate (Item 9) for the washing steps **to decrease the inter- and intra-assay coefficient of variations (CVs)**. The filter plate requires a vacuum manifold compatible with a standard 96-well microplate.

### **Additional Materials Required**

- Orbital 96-well plate shaker (with ability to reach 1000 rpm)
- Flow cytometer with blue and red lasers capable of measuring phycoerythrin (PE) and allophycocyanin (APC) channels
- Aluminum foil or 96-well Plate Aluminum Sealers
- Distilled water
- 1.5 ml polypropylene microcentrifuge tubes or similar
- Microcentrifuge
- **Recommended**: Vacuum manifold compatible with a 96-well microplate if using the Filter 96-Well Microplate (Item 10)
- Optional: 96-well round bottom plate (to prepare samples)
- 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  $\mu$ l of diluted serum or plasma, cell culture media, or other fluids. Actual sample dilutions may need to be empirically determined.
- It's recommended to use a 96-well round bottom plate to prepare samples before transferring the samples to the test plate or test tubes.
- We recommend analyzing samples at least in duplicate.
  
- **Serum Samples**
  - **Serum samples: Dilute samples 2-fold with PBS** (e.g., dilute 30  $\mu$ l of serum with 30  $\mu$ l of PBS), **which is equal to 4-fold total dilution after mixed with beads.**
  - If further dilution is needed, **RayBio<sup>®</sup> Serum Diluent** (Item 4) should be used.
  
- **Cell Culture Supernatant / Conditioned Media**
  - **Samples: equal to 2-fold total dilution after mixed with beads.**
  - The use of serum (e.g., fetal bovine serum; FBS) in cell culture can result in high background. To learn how to prepare samples, see our [Tips on Sample Preparation here: https://www.raybiotech.com/tips-on-sample-preparation/](https://www.raybiotech.com/tips-on-sample-preparation/).
  - If cell culture supernatant from serum-containing conditioned media is tested, it is highly recommended that complete medium be used as a negative control since many types of sera contain proteins that may cross-react with the antibodies.

**Note:** Levels of target protein(s) may vary between different samples. Optimal dilution factors for each sample must be determined empirically by the investigator.

**Note:** Matrix effects are a common cause of non-linear dilution responses. This can occur when proteins or other components within the sample affect the immunoreactivity of the target molecule. These matrix components can also affect the ability of the antibody to recognize its target within the sample. Auto-antibodies, binding proteins, hemolysis, or certain disease states can contribute to this phenomenon. If matrix effects are suspected, centrifuge the sample(s), and dilute further such that matrix effects become negligible.

**Note:** 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.
  - Protect fluorescent multiplex beads from light.
1. Dilute and prepare only what is needed to perform the tests for each experiment.
  2. Vortex the RayPlex Multiplex Bead Cocktail (Item 1) for 30 seconds before use. Use 25  $\mu$ l beads per test.
  3. Dilute the 20X Wash Buffer (Item 3) 20-fold with de-ionized (DI) H<sub>2</sub>O to prepare 1X Wash buffer (See Example note below).
  4. Dilute the 10X Streptavidin-PE (Item 7) 10-fold in 1X Assay Diluent (Item 2) to prepare a 1X Streptavidin-PE working stock. Use 50  $\mu$ l per test.

**Example:** A 20-fold dilution would be 1-part 20X Wash Buffer and 19 parts DI H<sub>2</sub>O.

### C. Preparation of Protein Standards

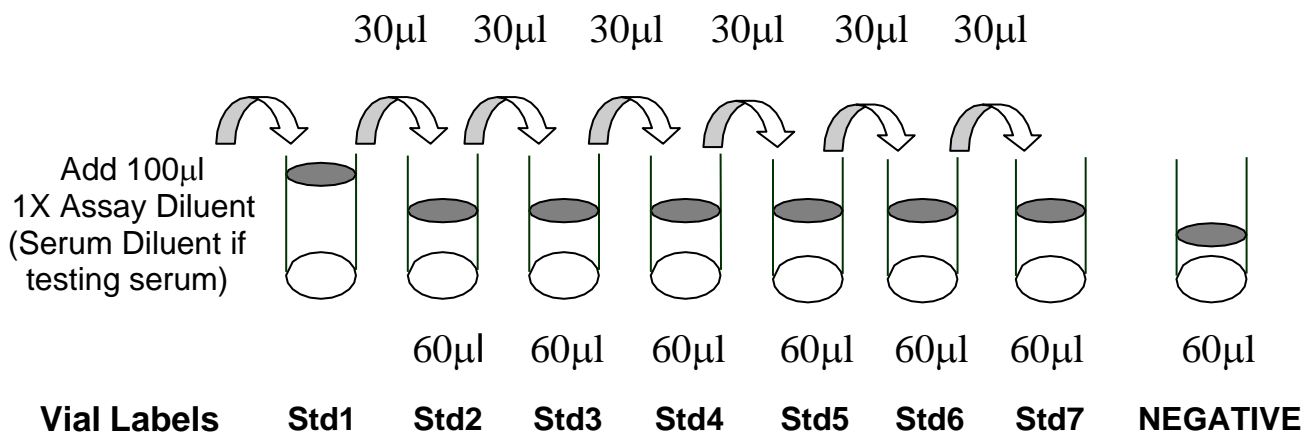
1. Centrifuge the Lyophilized Protein Standard Mix (Item 5) briefly (1-3 seconds) to collect contents to the bottom of the vial.
2. Reconstitute the Lyophilized Protein Standard Mix (Item 5):
  - For **conditioned media or non-serum fluids** add 100 $\mu$ l 1X Assay Diluent (Item 2) to the tube. Dissolve the powder thoroughly and gently by pipetting up and down 5-10 times. Label this tube as Std1.
  - For **serum**, add 100 $\mu$ l RayBio<sup>®</sup> Serum Diluent (Item 4) to the tube. Dissolve the powder thoroughly and gently by pipetting up and down 5-10 times. Label this tube as Std1.

**Note:** Use the Lyophilized Protein Standard Mix (Item 5) within 1 hour of preparation.

**Note:** When testing **serum**, RayBio<sup>®</sup> Serum Diluent (Item 4) should be used to reconstitute and serially dilute the standard mixture.

3. Obtain 6 clean 1.5 ml microcentrifuge tubes (or use a 96-well round-bottom plate). Label the tubes/wells as Std2 to Std7. Add 60  $\mu$ l 1X Assay Diluent (Item 2), or RayBio<sup>®</sup> Serum Diluent (item 4) as applicable to each tube.
4. Pipette 30  $\mu$ l Std1 into tube Std2 and mix gently. Perform 5 more serial dilutions by adding 30  $\mu$ l of Std2 to tube Std3, mix, and so on. (See also image below)

#### Prepare serial dilutions of protein standards



5. Add 60  $\mu$ l 1X Assay Diluent (Item 2) or RayBio<sup>®</sup> Serum Diluent (item 4) to another tube labeled as NEGATIVE. Do not add standard or samples to the NEGATIVE tube. This tube will be used as the negative control.

**Note:** The exact concentrations of each standard protein for the Std1 to Std7 serial dilutions in this kit can be found in Section VII.

## D. Assay Procedure

1. Prepare a V-bottom 96-well Microplate (Item 9) or 1.2µm Filter 96-well Microplate (Item 10) and mark positions for the standards and samples. Duplicate tests are recommended for all standards and samples.

**Note:** RayBiotech recommends using a Filter 96-well Microplate (Item 10) in lieu of the V-bottom 96-well microplate (Item 9) for the washing steps to decrease the inter- and intra-assay coefficient of variations (CVs). The filter plate requires a vacuum manifold compatible with a standard 96-well microplate.

2. Add 25 µl of RayPlex Multiplex Bead Cocktail (Item 1) to each well that will contain the NEGATIVE, Standard, or Sample.
3. Add 25 µl of the NEGATIVE, Standard, or Sample to the appropriate well. The total volume in each well is now 50 µl (**serum sample is diluted 4-fold totally with beads in 50 µl reaction system, cell culture supernatant / conditioned media is diluted 2-fold totally with beads**). Place plate on an orbital plate shaker. Shake at 1000 rpm at room temperature for 2 hours.

**Note:** This step can also be performed overnight at 4°C, which may increase the signal-to-noise ratio, particularly for proteins with low concentrations. The optimal incubation length (i.e., room temperature for 2 hours or overnight at 4°C) for the experiment must be determined empirically by the investigator.

4. Wash the beads by adding 200 µl 1X Wash Buffer (*diluted* Item 3; See Section B).
  - **V-bottom 96-well Microplate (Item 9):** Spin the V-bottom microplate down at 1000 g for 5 minutes at room temperature and remove the supernatant using a multichannel pipet.
  - **Filter 96-well Microplate (Item 10):** Turn on the vacuum, open the valve, and place the filter plate on the vacuum. Do not allow pressure to exceed 10 inches Hg (254 mm Hg). Close the valve and remove the plate when the wells have drained.

Repeat this wash step one more time.

5. Add 25 µl of 1X Biotinylated Detection Antibody Cocktail (Item 6) to each well. Resuspend the beads by gently pipetting and incubate on an orbital shaker at 1000 rpm at room temperature for 1 hour.

6. Wash plate twice as outlined in Step 4.
7. Add 50  $\mu$ l of 1X Streptavidin-PE (*diluted* Item 7; See Section B) to each well, incubate on an orbital shaker at 1000 rpm at room temperature for 30 minutes.
8. Wash plate once as outlined in Step 4.
9. Resuspend in 150  $\mu$ l of 1X Wash Buffer (*diluted* Item 3; See Section B).
10. Prepare samples for analysis on a flow cytometer:
  - Automated High Throughput Sampler (HTS): if using a filter plate, transfer samples to the V-bottom 96-well Microplate (Item 9) or any plate compatible with your HTS.
  - Manual reading: transfer samples to compatible tubes.
11. Analyze samples on a flow cytometer (see Section E).

### **E. Flow Cytometer Set-up and Data Acquisition**

**NOTE:** The RayPlex Mouse Chemokines Array 1 requires a flow cytometer that is equipped with blue and red lasers capable of detecting fluorescence in the PE and APC channels, respectively.

**NOTE:** Perform standard QC and optimization steps to prepare the flow cytometer, then set up the flow cytometer as you would for normal use. Manual adjustment of the PE and APC compensation may be necessary if the APC fluorescence is too intense, which sometimes causes a smiling effect (curved population grouping).

**NOTE:** These instructions are general for all RayPlex assays. The target count, bead size, and populations may differ from the assay above. Please see your assay's specific manual Section IV and VII for the bead size and populations present in your array.

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

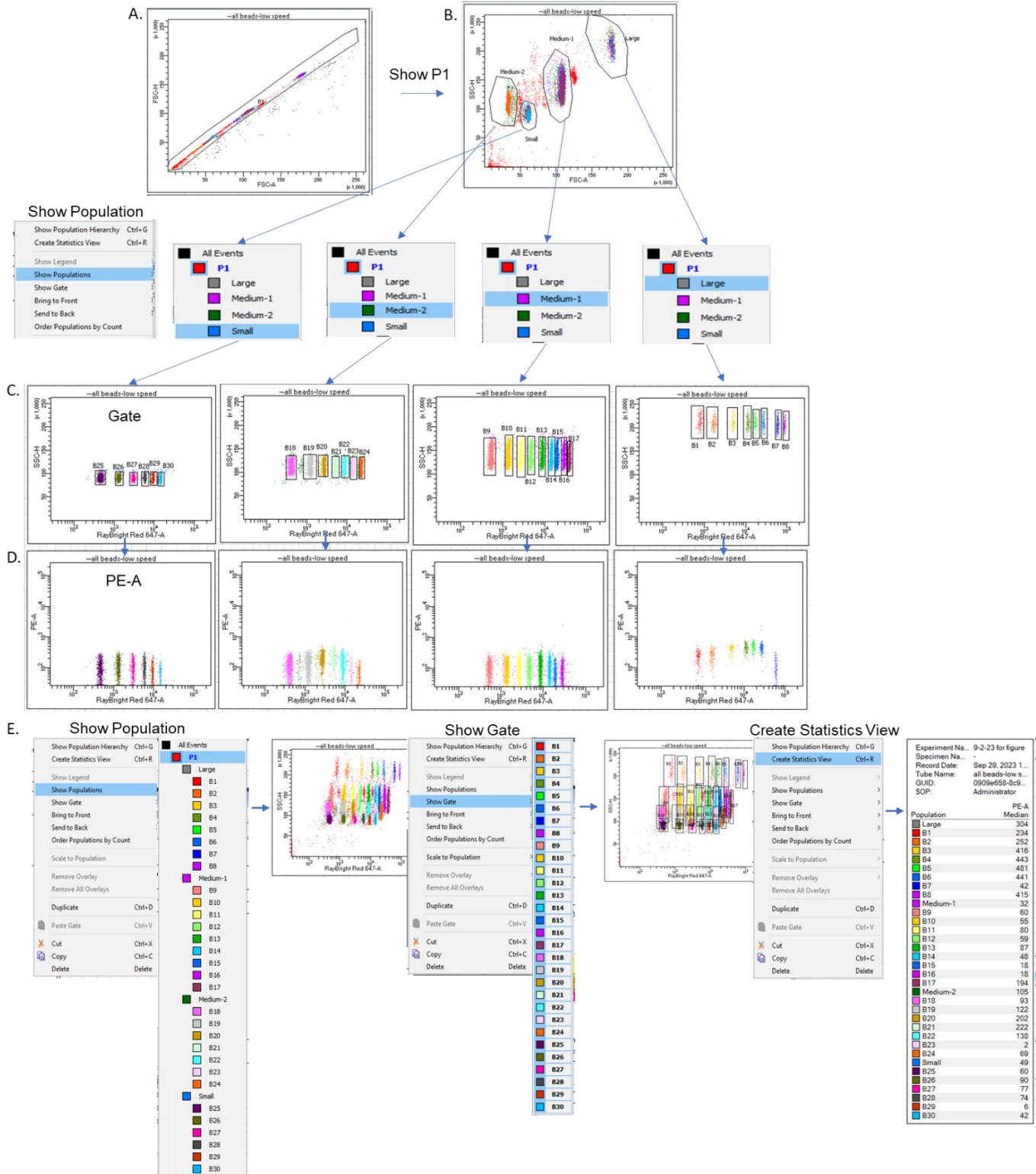
**Note:** The Quality Controls beads are fluorescent particles that are used for flow cytometer setup, e.g., CS&T beads for a BD flow cytometer.

2. Start a new experiment with PE and APC channels.
3. Resuspend the provided Flow Cytometer Setup Bead Cocktail (Item 8) in 200  $\mu$ l 1x Wash Buffer (*diluted* Item 3) and run them on the flow cytometer.
4. Create an FSC-H/FSC-A daughter population for “Single beads” selection to remove and limit doublets or higher complexes (Figure 3A).
5. Create a new dot plot from the “Single beads” parent gate, adjust voltage for FSC (forward scatter, linear mode) and SSC (side scatter, linear mode) so that the small, medium, and/or large bead populations are clearly defined. Then create a small, medium, and/or large beads gate (Figure 3B).
6. Create a new dot plot from the small, medium, and/or large 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 and well separated throughout in a prominent area (Figure 3C).
7. Create a dot plot of APC versus PE (using log scale for both) from the small, medium, and/or large beads’ parent populations. Run a small amount of beads from the NEGATIVE control. Adjust the PE voltage so that the PE mean fluorescent intensity (MFI) is around  $10^{2-3}$  for each bead population.

**Note:** *To keep the testing consistent for each assay, we recommend use of Rainbow Particles, Rainbow Beads, or similar to standardize the assay if they are run prior to every analysis. The optimized MFI for both the APC and PE channels for the Rainbow Particles allow the experiment to be consistent from time to time and machine to machine.*

8. Set the number of bead events to be acquired to at least 200 per target population for best results. More beads will improve data accuracy.
9. Following setup, run the standards and samples (Figure 3D).
10. Create a statistical view to show the PE MFI for each population (Figure 3E). The MFI of all analytes and samples can be exported in Excel format by “Batch Analysis” of a whole specimen in FACSDiva. Researchers can also export raw data as “FCS” files and analyze in FlowJo software.





**Figure 3. Representative Images of Gated Flow Cytometry Scatter Plots Using RayPlex Arrays with small, medium-1, medium-2, and large beads. (A) Single beads are (B) separated by size, then by (C) dye concentration. (D) Beads are read based on PE-A concentration. (E) All raw PE-A Median values based on Gate of (C).**

*Note: The above figures are for demonstration purposes only. This kit may not contain all bead populations shown.*

## **F. Data Analysis**

1. The MFI of all analytes and samples can be exported in Excel format by “Batch Analysis” of a whole specimen in FACSDiva (See **Appendix: RayPlex<sup>®</sup> Data Analysis Using BD FACS Diva Batch Analysis**).
2. MFI data of each analyte for all samples can also be acquired by exporting the entire dataset as an FCS file. Analyze data in FlowJo or equivalent software and export the PE MFI into an Excel (or similar) format.

## VII. Assay Information

### 1. 8-Point Standard Protein Concentrations (pg/mL)

The concentrations of target proteins corresponding to standard (Std) 1-7 serial dilutions are displayed below in pg/mL. These concentrations are used to produce a standard curve.

| Target                          | STD1     | STD2    | STD3    | STD4   | STD5   | STD6  | STD7  | CTRL |
|---------------------------------|----------|---------|---------|--------|--------|-------|-------|------|
| <b>MCP-1</b>                    | 50000.0  | 16666.7 | 5555.6  | 1851.9 | 617.3  | 205.8 | 68.6  | 0.0  |
| <b>I-TAC</b>                    | 10000.0  | 3333.3  | 1111.1  | 370.4  | 123.5  | 41.2  | 13.7  | 0.0  |
| <b>MIP-1<math>\beta</math></b>  | 100000.0 | 33333.3 | 11111.1 | 3703.7 | 1234.6 | 411.5 | 137.2 | 0.0  |
| <b>Fractalkine</b>              | 200000.0 | 66666.7 | 22222.2 | 7407.4 | 2469.1 | 823.0 | 274.3 | 0.0  |
| <b>TARC</b>                     | 100000.0 | 33333.3 | 11111.1 | 3703.7 | 1234.6 | 411.5 | 137.2 | 0.0  |
| <b>MDC</b>                      | 10000.0  | 3333.3  | 1111.1  | 370.4  | 123.5  | 41.2  | 13.7  | 0.0  |
| <b>KC</b>                       | 20000.0  | 6666.7  | 2222.2  | 740.7  | 246.9  | 82.3  | 27.4  | 0.0  |
| <b>Eotaxin-1</b>                | 2000.0   | 666.7   | 222.2   | 74.1   | 24.7   | 8.2   | 2.7   | 0.0  |
| <b>MIG</b>                      | 30000.0  | 10000.0 | 3333.3  | 1111.1 | 370.4  | 123.5 | 41.2  | 0.0  |
| <b>RANTES</b>                   | 100000.0 | 33333.3 | 11111.1 | 3703.7 | 1234.6 | 411.5 | 137.2 | 0.0  |
| <b>BLC</b>                      | 100000.0 | 33333.3 | 11111.1 | 3703.7 | 1234.6 | 411.5 | 137.2 | 0.0  |
| <b>MIP-3<math>\alpha</math></b> | 70000.0  | 23333.3 | 7777.8  | 2592.6 | 864.2  | 288.1 | 96.0  | 0.0  |

### 2. Representation of Standard Curve MFI Values

The values in this table are an example of MFI values that can be used to generate a standard curve. These values are for demonstration purposes only. A standard curve must be run with each assay since the MFI values will vary from experiment to experiment.

|                | MCP-1   | I-TAC   | MIP-1 $\beta$ | Fractalkine | TARC     | MDC     | KC      | Eotaxin-1 | MIG     | RANTES   | BLC      | MIP-3 $\alpha$ |
|----------------|---------|---------|---------------|-------------|----------|---------|---------|-----------|---------|----------|----------|----------------|
| Std 1 (pg/ml)  | 50000.0 | 10000.0 | 100000.0      | 200000.0    | 100000.0 | 10000.0 | 20000.0 | 2000.0    | 30000.0 | 100000.0 | 100000.0 | 70000.0        |
| Standard 1 MFI | 9855.7  | 12877.0 | 20708.7       | 6635.0      | 29282.7  | 12949.0 | 10625.7 | 12680.7   | 8141.3  | 13222.3  | 25911.3  | 10520.7        |
| Standard 2 MFI | 5671.7  | 9302.3  | 12158.0       | 6075.7      | 17759.0  | 7695.3  | 5583.3  | 7875.0    | 4242.7  | 9543.7   | 13681.7  | 6975.3         |
| Standard 3 MFI | 2815.3  | 4521.7  | 9238.7        | 3960.3      | 10039.7  | 2698.7  | 1932.7  | 4388.0    | 1895.0  | 5171.3   | 3876.3   | 4057.3         |
| Standard 4 MFI | 1388.0  | 2355.0  | 6687.0        | 2774.7      | 5118.7   | 1009.3  | 794.0   | 2713.3    | 993.3   | 2281.7   | 1560.7   | 2359.3         |
| Standard 5 MFI | 736.7   | 1476.0  | 4067.0        | 1319.0      | 2298.0   | 621.3   | 478.3   | 1844.3    | 590.3   | 784.0    | 730.3    | 1102.3         |
| Standard 6 MFI | 598.0   | 1280.7  | 2186.7        | 1154.7      | 1345.3   | 507.3   | 405.3   | 1144.3    | 440.7   | 268.7    | 517.0    | 661.7          |
| Standard 7 MFI | 580.3   | 1350.7  | 1075.7        | 916.3       | 915.0    | 470.7   | 397.3   | 1058.7    | 437.0   | 138.3    | 460.0    | 437.7          |
| Negative MFI   | 500.7   | 909.0   | 652.7         | 893.7       | 575.3    | 407.0   | 338.0   | 695.3     | 361.7   | 62.7     | 377.3    | 245.3          |

### 3. Lower Limit of Detection (LOD) of Target Proteins

The LOD is the minimum detectable concentration (i.e., sensitivity) of the assay. It was determined by spiking each purified protein standard into RayBio® Serum Diluent (Item 4).

| Target         | LOD (pg/mL) |
|----------------|-------------|
| MCP-1          | 339.8       |
| I-TAC          | 85.3        |
| MIP-1 $\beta$  | 6.6         |
| Fractalkine    | 774.2       |
| TARC           | 194.7       |
| MDC            | 154.5       |
| KC             | 229.7       |
| Eotaxin-1      | 4.8         |
| MIG            | 146.5       |
| RANTES         | 43.8        |
| BLC            | 1047.8      |
| MIP-3 $\alpha$ | 35.4        |

**Note:** The LOD may vary from run-to-run and batch-to-batch.

### 4. Cross Reactivity

No detectable or significant cross reactivity was detected between antibodies or targets.

## 5. Percent (%) Recovery of Spiked Standards

For spike recovery of standards, target proteins with concentrations corresponding to standard 2 (Std2) were spiked into RayBio® Serum Diluent (Item 4) across 9 replicates. The spiked proteins were assayed, and the measured concentrations were compared to the expected concentrations.

Ray Bio® Mouse Serum Diluent

| Target         | Percent Recovery |
|----------------|------------------|
| MCP-1          | 98.8%            |
| I-TAC          | 100.0%           |
| MIP-1 $\beta$  | 100.1%           |
| Fractalkine    | 99.1%            |
| TARC           | 97.2%            |
| MDC            | 99.0%            |
| KC             | 99.1%            |
| Eotaxin-1      | 101.3%           |
| MIG            | 99.8%            |
| RANTES         | 102.2%           |
| BLC            | 100.4%           |
| MIP-3 $\alpha$ | 100.8%           |

## 6. Linearity of Dilution

To test the linearity of dilution, samples (n = 12) were serially diluted in RayBio® Serum Diluent (Item 4) 1:3, 1:9, and 1:21. The measured concentrations of serially diluted samples were compared to that of the undiluted samples and averaged. See data below for data.

RayBio® Mouse Serum Diluent

| Target         | Linearity of Dilution |
|----------------|-----------------------|
| MCP-1          | 98.3%                 |
| I-TAC          | 101.9%                |
| MIP-1 $\beta$  | 114.8%                |
| Fractalkine    | 111.2%                |
| TARC           | 100.4%                |
| MDC            | 100.5%                |
| KC             | 100.6%                |
| Eotaxin-1      | 102.0%                |
| MIG            | 101.3%                |
| RANTES         | 102.6%                |
| BLC            | 103.0%                |
| MIP-3 $\alpha$ | 103.7%                |

## 7. Intra- and Inter-Assay Precision

### 1) Intra-Assay Precision

Spiked standard protein in RayBio® Serum Diluent (Item 4) was analyzed in triplicate. This was performed at two different concentrations per standard protein (i.e., Sample #2, Sample #3 or Sample #3, Sample #4). The intra-assay precision, or coefficient of variation (CV), of the samples is provided below.

| Target         | Sample # | Mean (pg/mL) | Standard Deviation | CV %  |
|----------------|----------|--------------|--------------------|-------|
| MCP-1          | 2        | 16662.6      | 318.8              | 1.9%  |
|                | 3        | 5563.9       | 344.0              | 6.2%  |
| I-TAC          | 2        | 3500.5       | 350.9              | 10.0% |
|                | 3        | 1010.2       | 240.8              | 23.8% |
| MIP-1 $\beta$  | 2        | 26384.2      | 2819.0             | 10.7% |
|                | 3        | 12859.7      | 839.9              | 6.5%  |
| Fractalkine    | 3        | 19845.5      | 394.8              | 2.0%  |
|                | 4        | 9285.7       | 1437.6             | 15.5% |
| TARC           | 2        | 32692.8      | 242.7              | 0.7%  |
|                | 3        | 11466.9      | 205.0              | 1.8%  |
| MDC            | 2        | 3348.3       | 242.4              | 7.2%  |
|                | 3        | 1087.8       | 40.9               | 3.8%  |
| KC             | 2        | 6697.3       | 543.3              | 8.1%  |
|                | 3        | 2158.3       | 39.6               | 1.8%  |
| Eotaxin-1      | 3        | 204.3        | 7.0                | 3.4%  |
|                | 4        | 73.4         | 8.7                | 11.8% |
| MIG            | 3        | 3119.4       | 240.5              | 7.7%  |
|                | 4        | 1151.4       | 151.6              | 13.2% |
| RANTES         | 3        | 11042.3      | 1497.1             | 13.6% |
|                | 4        | 3839.1       | 405.6              | 10.6% |
| BLC            | 2        | 33524.3      | 3211.8             | 9.6%  |
|                | 3        | 10581.7      | 713.0              | 6.7%  |
| MIP-3 $\alpha$ | 2        | 23561.8      | 1881.6             | 8.0%  |
|                | 3        | 7539.7       | 788.6              | 10.5% |

## 2) Inter-Assay Precision

Spiked standard protein in RayBio® Serum Diluent (Item 4) was analyzed in triplicate across three independent assays. This was performed at two different concentrations per standard protein (i.e., Sample #2, Sample #3 or Sample #3, Sample #4). The inter-assay precision in (CV) of the samples is provided below.

| Target         | Sample # | Mean (pg/mL) | Standard Deviation | CV %  |
|----------------|----------|--------------|--------------------|-------|
| MCP-1          | 2        | 16573.7      | 100.8              | 0.6%  |
|                | 3        | 5740.4       | 159.6              | 2.8%  |
| I-TAC          | 2        | 3358.5       | 197.8              | 5.9%  |
|                | 3        | 1135.1       | 121.4              | 10.7% |
| MIP-1 $\beta$  | 2        | 27157.9      | 726.0              | 2.7%  |
|                | 3        | 12477.1      | 343.0              | 2.7%  |
| Fractalkine    | 3        | 21891.6      | 1907.3             | 8.7%  |
|                | 4        | 8216.8       | 1094.9             | 13.3% |
| TARC           | 2        | 32480.5      | 416.4              | 1.3%  |
|                | 3        | 11673.8      | 458.1              | 3.9%  |
| MDC            | 2        | 3345.7       | 3.9                | 0.1%  |
|                | 3        | 1099.8       | 14.9               | 1.4%  |
| KC             | 2        | 6686.4       | 21.7               | 0.3%  |
|                | 3        | 2194.4       | 49.0               | 2.2%  |
| Eotaxin-1      | 2        | 676.8        | 23.9               | 3.5%  |
|                | 3        | 214.9        | 18.7               | 8.7%  |
| MIG            | 2        | 10038.0      | 198.1              | 2.0%  |
|                | 3        | 3299.5       | 296.2              | 9.0%  |
| RANTES         | 2        | 34080.3      | 882.2              | 2.6%  |
|                | 3        | 10842.7      | 521.9              | 4.8%  |
| BLC            | 2        | 33820.7      | 277.1              | 0.8%  |
|                | 3        | 10612.4      | 31.1               | 0.3%  |
| MIP-3 $\alpha$ | 2        | 23534.2      | 928.8              | 3.9%  |
|                | 3        | 7428.1       | 553.0              | 7.4%  |

**Note: All data in Section VII were obtained using a filter plate. Five-parameter logistic curve fitting was used for generation of standard curve and calculation of concentrations.**

## VIII. Troubleshooting Guide

| Issue                      | Possible Causes  | Recommendations  |
|----------------------------|--|--|
| <b>Weak Signal</b>         | Low protein content in sample  | Increase the length of the sample-bead incubation to overnight at 4°C (see Section D, step 3).<br>Decrease sample dilution.<br><i>Note that even if the incubation is increased or the sample dilution is decreased, it is not guaranteed that the protein will be detected.</i> |
|                            | Detection antibody over diluted  | Increase detection antibody concentration.   |
|                            | Sample matrix effect   | Optimize sample dilution.<br>For serum samples, prepare the standards in the provided “RayBio® Serum Diluent” (Item 4).<br>Adjust the PE MFI using a small amount of beads from the negative control to around $10^{2-3}$ .  |
|                            | Improper storage of kit  | Store kit at suggested temperature and use within 6 months of receipt.   |
|                            | Reagent evaporation  | Cover the incubation plate with adhesive film during incubation.   |
| <b>Poor standard curve</b> | Cross-contamination from neighboring wells                                 | Avoid overflowing wells during wash steps.   |
|                            | Lyophilized Protein Standard Mix (Item 5) not properly prepared            | Serially dilute the Lyophilized Protein Standard Mix (Item 5) according to manual.   |
|                            | Lyophilized Protein Standard Mix (Item 5) degraded or not properly diluted | Reconstitute the Lyophilized Protein Standard Mix (Item 5) on ice <i>before</i> making serial dilutions.   |
| <b>High background</b>     | Improper flow cytometer setup and optimization                             | Run Setup Bead Cocktail (Item 8) before assay. Make sure top standard signal is not out of the linear range.   |
|                            | PE voltage PMT voltage is not set up correctly                             | Set PE voltage using a small amount of beads from the negative control so that the PE MFI is around $10^{2-3}$ .   |
|                            | Insufficient washing   | Increase wash time.<br>Use more wash buffer.   |



## IX. Appendix

### Data Analysis using Batch Analysis

Batch analysis allows exporting PE MFI data of each bead population directly from FACS Diva. Before batch analysis, it's important to create statistics view from the dot plot of gated beads populations and choose "PE-A Median" to be shown (Figure 4). Batch analysis can be done in either manual tube mode or HTS mode. Both methods for batch analysis are described below (Figure 5 and 6).

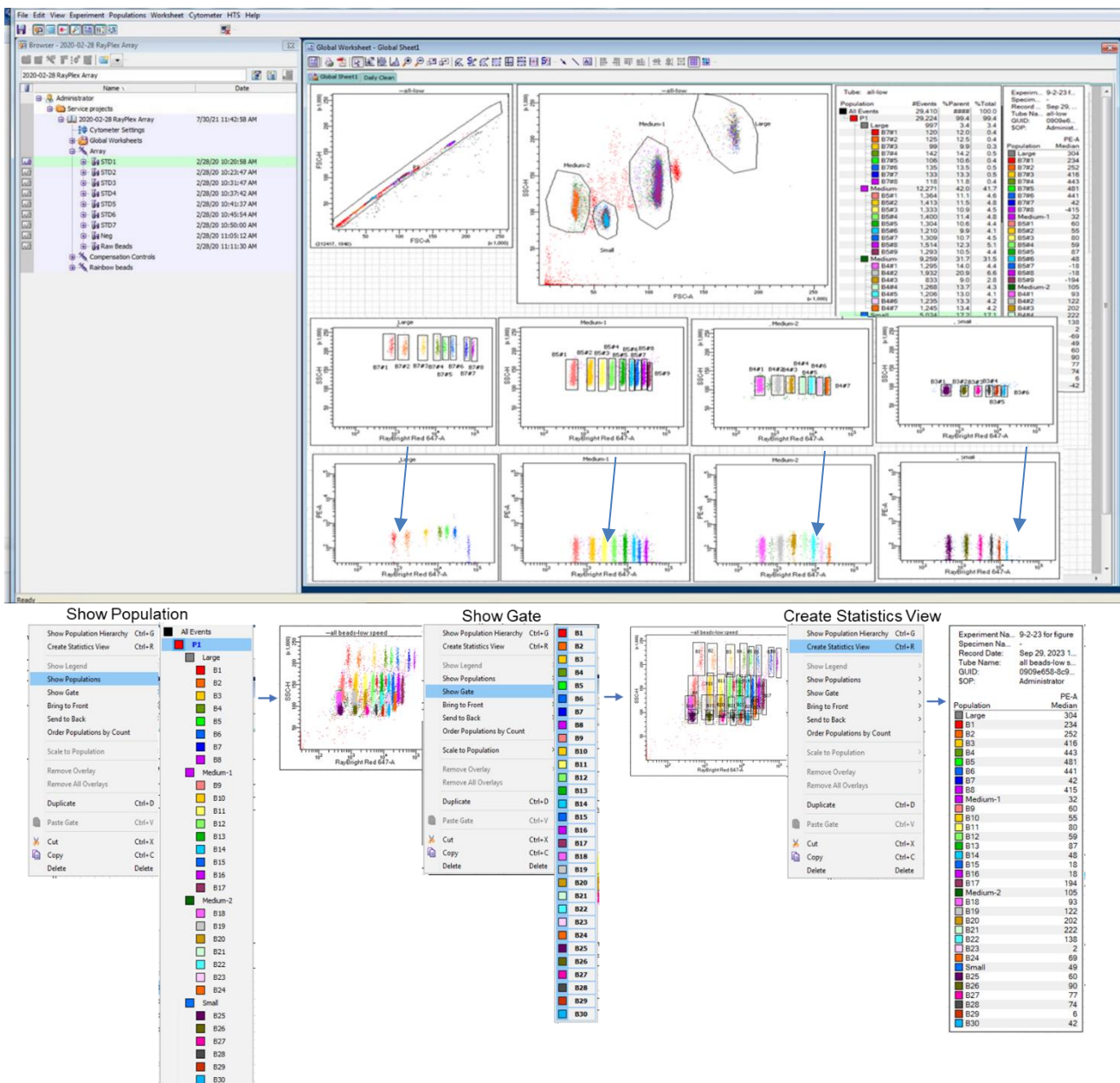


Figure 4. Screenshot of Creating Statistics View from the dot plot of gated beads populations. This is representative data only.

1. Create a dot plot by SSC-A (linear) and APC-A (log) to show all beads populations and make a gate for each population (see figure 4, “Gated Dot Plot”). Right click on the dot plot to “Create Statistics View”, then edit the statistics view so that “PE-A Median” of gated beads populations are shown (figure 4, “Statistics View of Gated Plot”).
2. Right click on the open folder containing your experiment data and select ‘Batch Analysis.’
3. When the pop-up appears, select the desired destination folder, and then hit start.

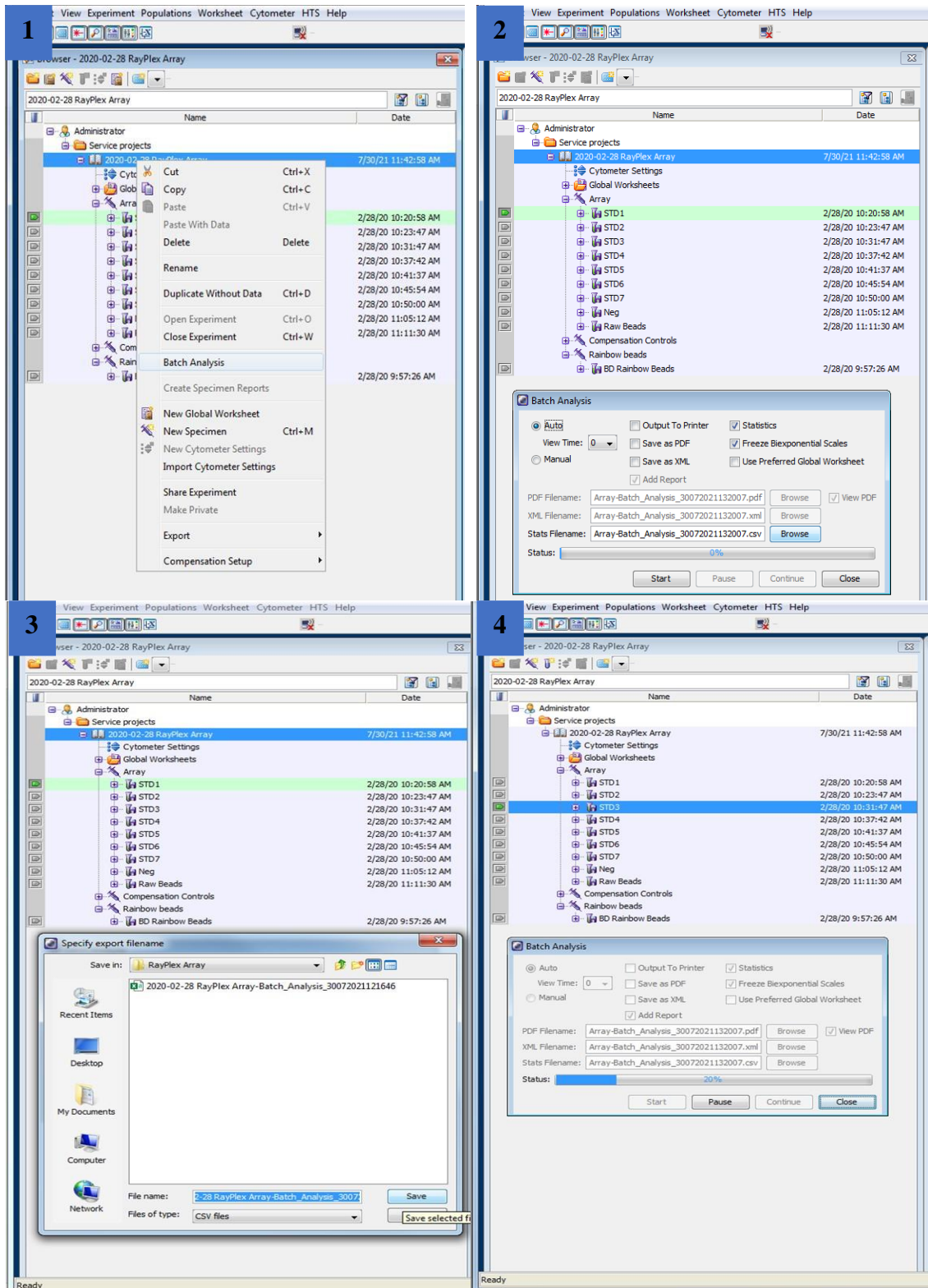
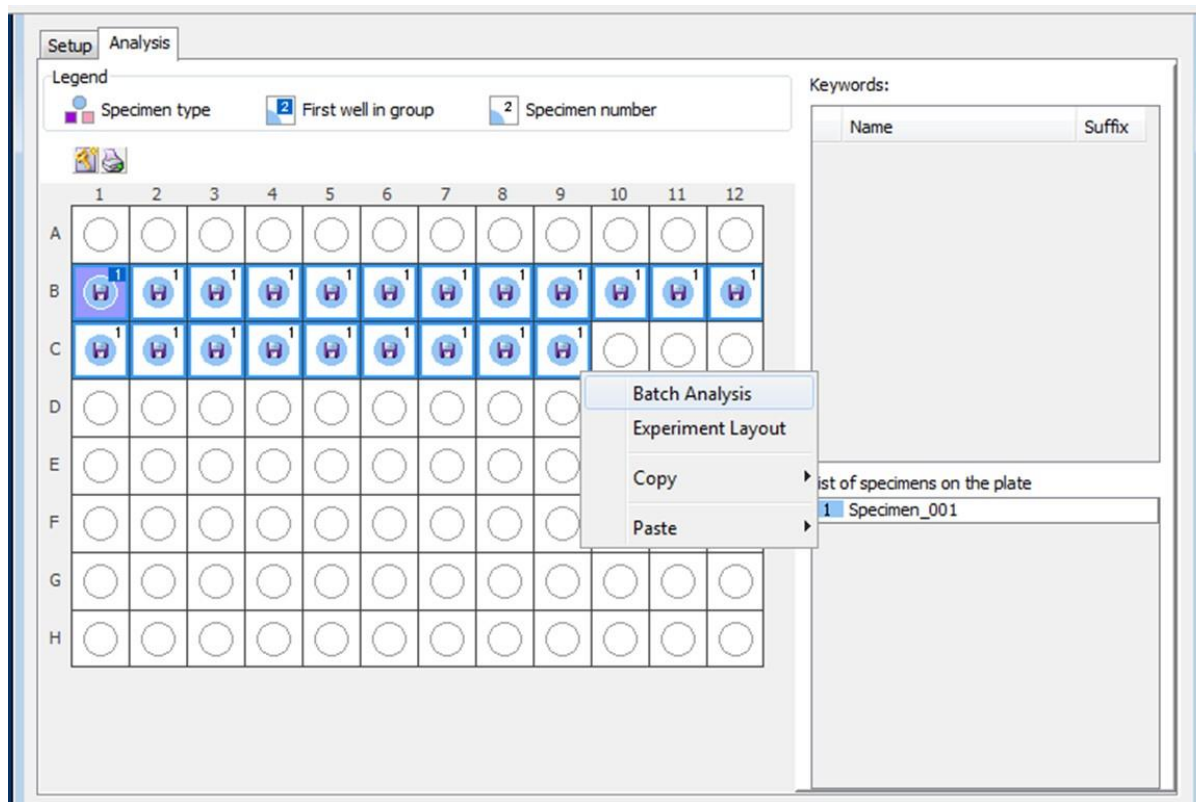


Figure 5. Screen shot showing how to begin the batch analysis through manual tube mode.



**Figure 6. Screen shot showing how to begin HTS batch analysis.**

4. When the HTS is done running samples, click on the 'Analysis' tab and highlight all of the samples. Right click and select 'Batch Analysis.' After selecting Batch Analysis through the HTS mode, the steps from Manual mode are the same.
5. Open the CSV file in the destination folder and select 'Save As' to save the data as an Excel workbook (Figure 7).

|    | A         | B              | C              | D              | E              | F              | G              | H              | I              | J               | K               | L               | M               | N |
|----|-----------|----------------|----------------|----------------|----------------|----------------|----------------|----------------|----------------|-----------------|-----------------|-----------------|-----------------|---|
| 1  | Tube Name | R1 PE-A Median | R2 PE-A Median | R4 PE-A Median | R5 PE-A Median | R6 PE-A Median | R7 PE-A Median | R8 PE-A Median | R9 PE-A Median | R10 PE-A Median | R12 PE-A Median | R13 PE-A Median | R14 PE-A Median |   |
| 2  | STD1      | 42203          | 35160          | 38647          | 48464          | 34527          | 28324          | 17515          | 15124          | 16826           | 14729           | 15436           | 16014           |   |
| 3  | STD2      | 24909          | 20453          | 22914          | 27861          | 20279          | 15538          | 10087          | 8766           | 10267           | 8787            | 9270            | 9982            |   |
| 4  | STD3      | 14359          | 11430          | 12524          | 16493          | 11076          | 7942           | 5942           | 4757           | 5882            | 5029            | 5248            | 5735            |   |
| 5  | STD4      | 7268           | 5314           | 5760           | 8796           | 5327           | 3730           | 3017           | 2098           | 2580            | 2201            | 2248            | 2607            |   |
| 6  | STD5      | 4158           | 2565           | 2781           | 4726           | 2554           | 1904           | 1461           | 963            | 1230            | 1027            | 1035            | 1226            |   |
| 7  | STD6      | 2639           | 1333           | 1437           | 3354           | 1327           | 1074           | 712            | 503            | 612             | 522             | 532             | 646             |   |
| 8  | STD7      | 1928           | 749            | 823            | 2339           | 835            | 682            | 393            | 286            | 360             | 305             | 318             | 392             |   |
| 9  | Neg       | 457            | 378            | 430            | 1676           | 456            | 377            | 182            | 134            | 174             | 140             | 143             | 206             |   |
| 10 | Raw Beads | 4              | 2              | 2              | 6              | 15             | 43             | 1              | 0              | 1               | 2               | 6               | 10              |   |
| 11 |           |                |                |                |                |                |                |                |                |                 |                 |                 |                 |   |
| 12 |           |                |                |                |                |                |                |                |                |                 |                 |                 |                 |   |
| 13 |           |                |                |                |                |                |                |                |                |                 |                 |                 |                 |   |
| 14 |           |                |                |                |                |                |                |                |                |                 |                 |                 |                 |   |
| 15 |           |                |                |                |                |                |                |                |                |                 |                 |                 |                 |   |

**Figure 7. Representative raw data obtained from batch analysis.** Rows = data from samples. Leftmost column = standard or sample name. (The names of beads are variable for different assays.)

## X. Notes

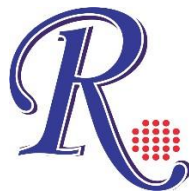
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