RayPlex® Human T Helper Cell Cytokine Array Kit

Quantitative Measurement of 13 Human Proteins by Flow Cytometry

Catalog numbers:
FAH-TH-1-48 (48 tests)
FAH-TH-1-96 (96 tests)
FAH-TH-1-192 (192 tests)
FAH-TH-1-480 (480 tests)

User Manual Version 5.0
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RayBiotech
Empowering your proteomics

ISO 13485:2016

Table of Contents

I.   Overview

Species	Human
Proteins Detected (13)	GM-CSF, IL-1β, IL-2, IL-4, IL-5, IL-6, IL-10, IL-13, IL-15, IL-17A, IL-22, IL-33, and TNF-α
Format	Bead-based
Detection Method	Flow Cytometry using a flow cytometer equipped with a blue laser (PE channel) and red laser (APC channel)
Minimal Sample Volume	25 µl per test after dilution. *The optimal dilution must be determined empirically by the researcher.*
Number of Replicates	At least 2 replicates. Each replicate is considered a test.
Reproducibility	See Section VIII (page 20) for inter-CV and intra-CV percentages for each protein.
Assay Duration	4 hours

II.   Introduction

T helper (Th) cells including Th1, Th2, Th9, Th17 and Th22 cells, play important roles in regulating immune responses, especially adaptive immune response. They secrete cytokines to stimulate various effector cells, such as cytotoxic T cells, B cells and macrophages. These cytokines are also involved in inflammatory and autoimmune diseases.

RayPlex Human Th Cytokine Array kit is a bead-based multiplex assay panel, using fluorescence-encoded beads to detect the above cytokines with a flow cytometer. This panel allows simultaneous quantification of 13 human cytokines, including IL-1β, 2, 4, 5, 6, 10, 13, 15, 17A, 22, 33, GM-CSF, and TNF-α, which are secreted by either or all Th1, Th2, Th9, Th17, and Th22. The assay is fully quantitative 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 to 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.

1 SAMPLE PROTEINS + ANTIBODY-COATED FLUORESCENT BEADS

2 ADD BIOTINYLATED DETECTION ANTIBODIES

3 ADD PE-CONJUGATED STREPTAVIDIN

4 FLOW CYTOMETRY

5 DATA ANALYSIS
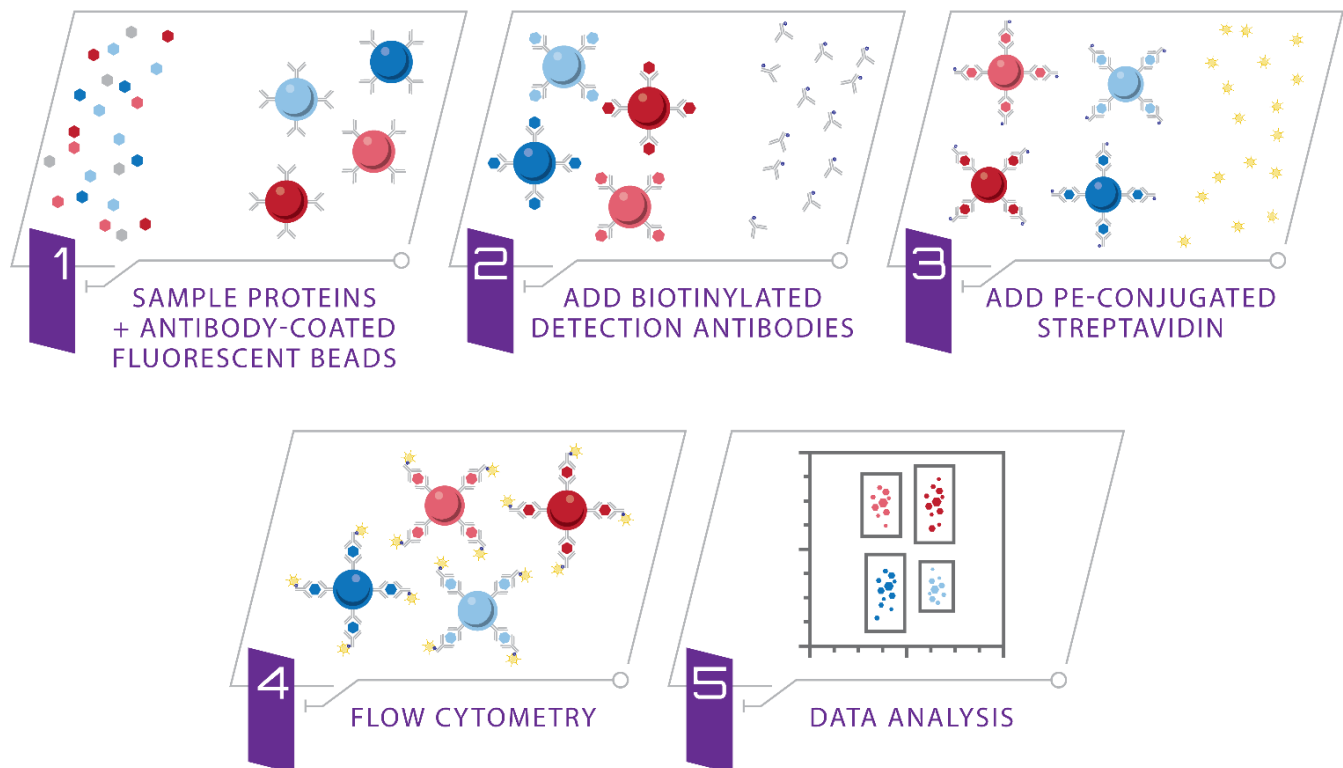
A schematic showing how RayPlex enables multiplex protein detection with flow cytometry.

IV. Bead ID and Distribution

1. General Overview of RayPlex Multiplex Beads.

As many as three different sizes of beads are used with RayPlex arrays: small, medium, and large, (Figures 1 and 2). This RayPlex Human T Helper Cell Cytokine Array Kit uses **medium** and **large** beads.
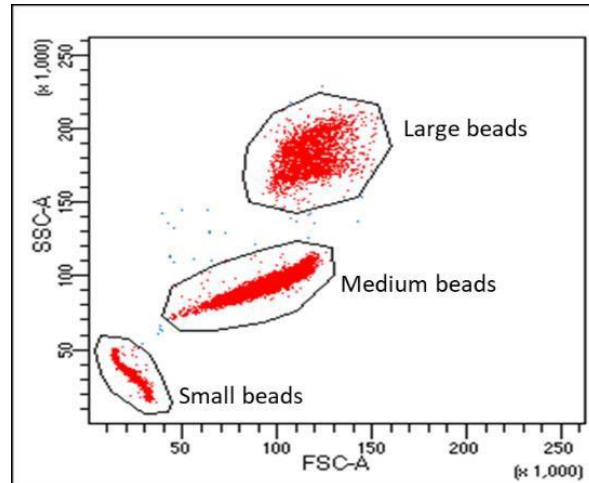

Figure 1. RayPlex uses different size beads
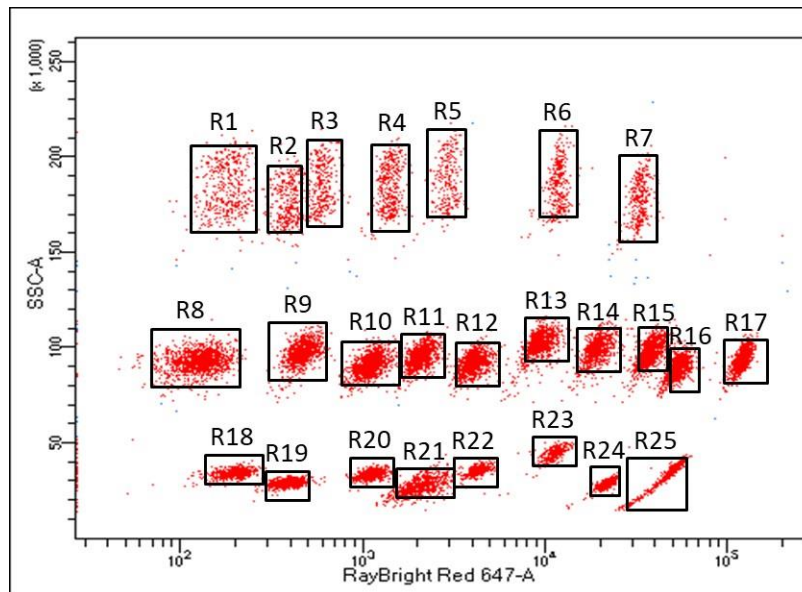
Figure 2. Different populations of bead size and color combinations ("R#") enable multiplex protein detection with RayPlex arrays. *Bead ID* R1 - R7: Large Beads. *Bead ID* R8 - R17: Medium Beads. *Bead ID* R18 - R25: Small Beads.

2. Target Proteins and associated Bead ID in this kit

FAH-TH-1	
R1	IL-13
R2	IL-22
R4	GM-CSF
R5	TNF-α
R6	IL-5
R7	IL-4
R8	IL-6
R10	IL-10
R11	IL-15
R12	IL-33
R13	IL-17A
R14	IL-1β
R15	IL-2
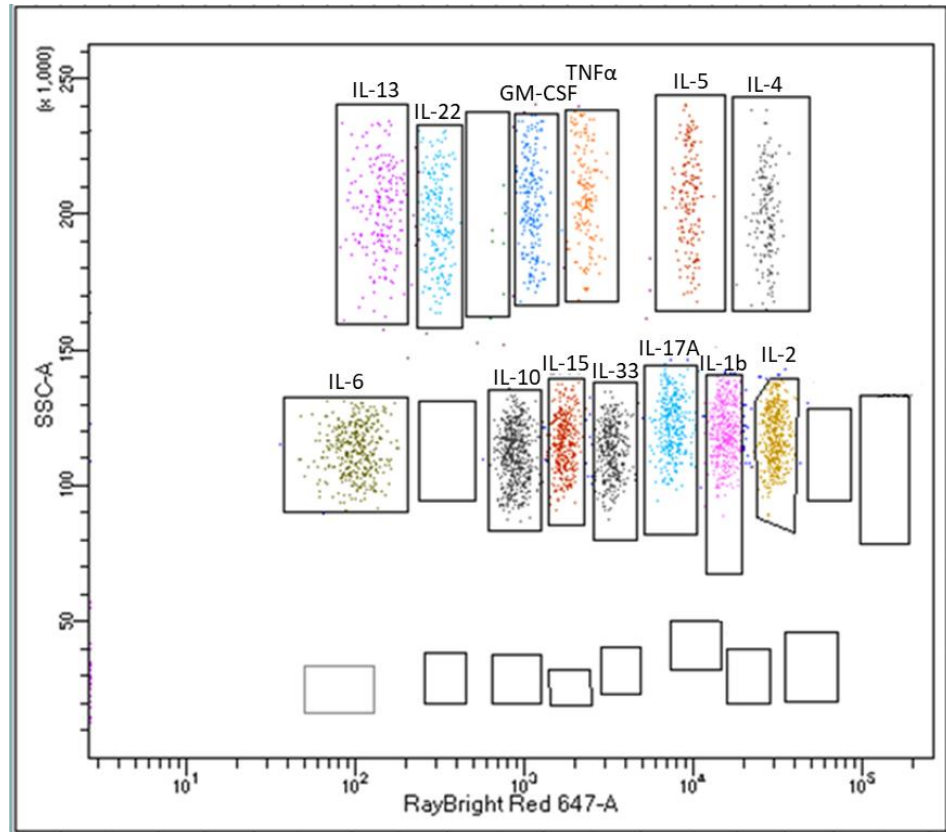
V.   Materials Provided

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 immediatelyafter preparation.* Additional vials of the Lyophilized Protein Standard Mix (Item 5) can be purchased separately (cat # FAH-TH-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
FAH-TH-1-Item1	RayPlex Multiplex Bead Cocktail	1.25 ml x 1 vial	1.25 ml x **2** vials
FAX-AD-2-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
FAH-SD-2-Item4	RayBio® Serum Diluent	2.5 ml x 1 vial	2.5 ml x **2** vials
FAH-TH-1-Item5	Lyophilized Protein Standard Mix	1	**2**
FAH-TH-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
FAH-TH-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:

1. *** 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.*
2. *RayBio® Serum Diluent (Item 4) is a pool of normal human 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 if a large lot of RayBio® Serum Diluent is required.*
3. ***RayBiotech recommends using a Filter 96-well Microplate (Item 10)*** *in lieu of the V-bottom96-well microplate (Item 9) for the washing steps* ***to decrease the inter- and intra-assaycoefficient 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
- **<u>Recommended</u>**: 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. <u>Preparation of Samples</u>

- We recommend the following parameters for your samples: 25 µl 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**
    - o **Serum samples: Dilute samples 2-fold with PBS (**e.g., dilute 30 µl of serum with 30 µl of PBS), **which is equal to 4-fold total dilution after mixed with beads.**
    - o If further dilution is needed, **RayBio® Serum Diluent** (Item 4) should be used.

- **Cell Culture Supernatant / Conditioned Media**
    - o **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/).
    - o 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. <u>Preparation of Reagents</u>

- 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 µl beads per test.
3. Dilute the 20X Wash Buffer (Item 3) 20-fold with de-ionized (DI) H_2O 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 µl per test.

   Example: A 20-fold dilution would be 1-part 20X Wash Buffer and 19 parts DI H_2O.
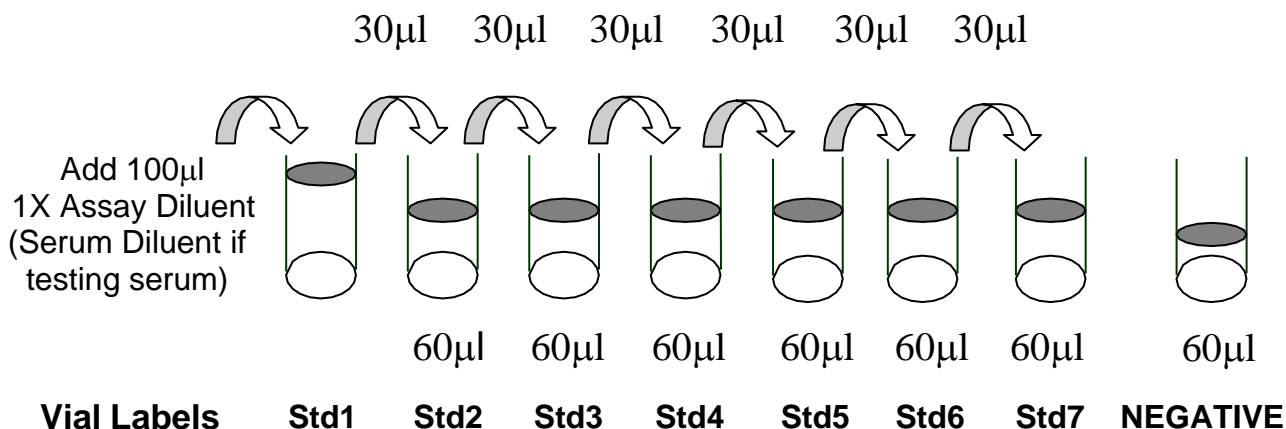
C. Preparation of Protein Standards

1. Centrifuge the Lyophilized Protein Standard Mix (Item 5) briefly (1-3 seconds) to collect contents to the bottle of the vial.

2. Reconstitute the Lyophilized Protein Standard Mix (Item 5);
   - For **conditioned media or non-serum fluids** add 100µl 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 100µl RayBio® 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® 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 µl 1X Assay Diluent (Item 2) or RayBio® Serum Diluent (item 4) as applicable to each tube.

4. Pipette 30 µl Std1 into tube Std2 and mix gently. Perform 5 more serial dilutions by adding 30 µl of Std2 to tube Std3, mix, and so on. (See also image below)

Prepare serial dilutions of protein standards

| | 30µl | 30µl | 30µl | 30µl | 30µl | 30µl | |

Add 100µl 1X Assay Diluent (Serum Diluent if testing serum)

| | | 60µl | 60µl | 60µl | 60µl | 60µl | 60µl | 60µl |
| **Vial Labels** | **Std1** | **Std2** | **Std3** | **Std4** | **Std5** | **Std6** | **Std7** | **NEGATIVE** |

5. Add 60 µl 1X Assay Diluent (Item 2) or RayBio® 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. <u>Assay Procedure</u>

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 µl 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 µl 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 high throughput sampler.
    - 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 Human T Helper Cell Cytokine 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 Sections 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 μl 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 $10^{2\sim3}$ 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.
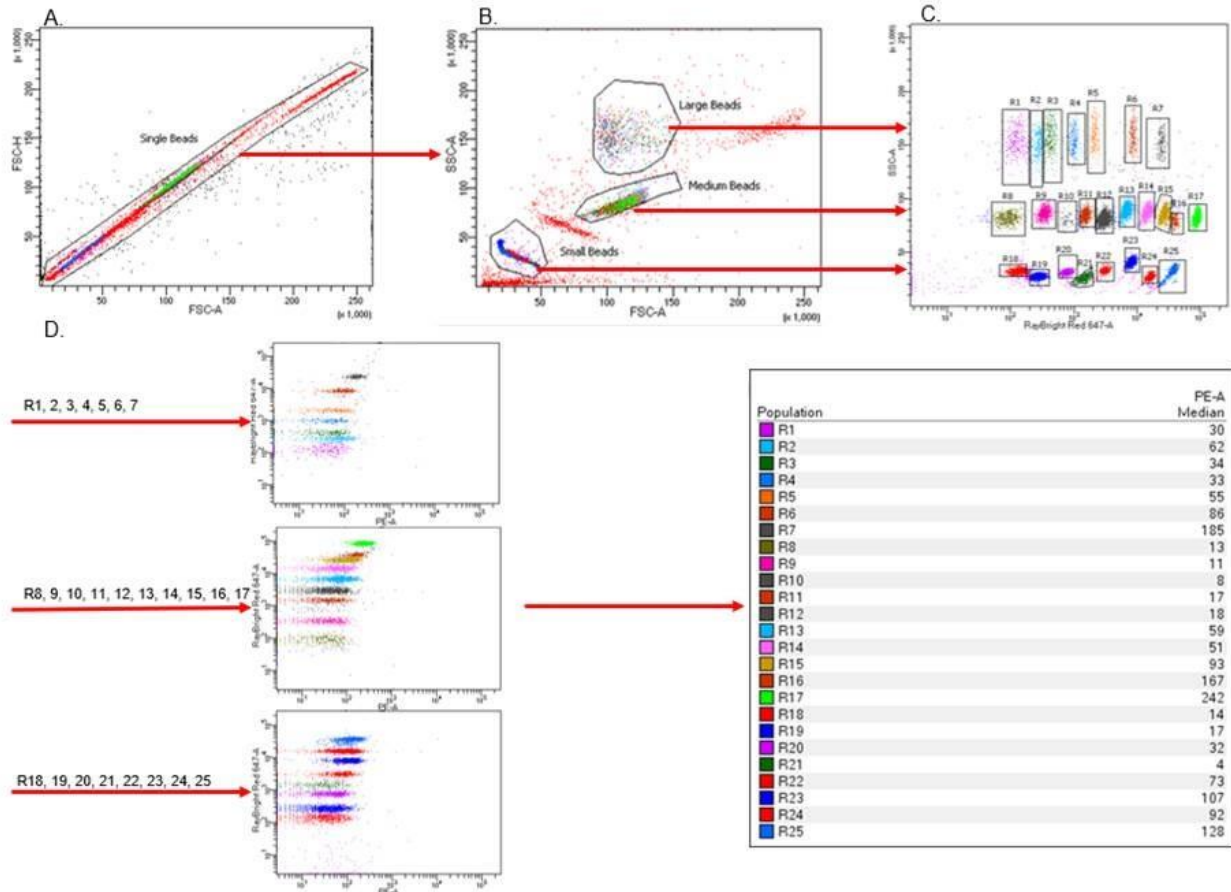
Figure 3. Representative Images of Gated Flow Cytometry Scatter Plots Using RayPlex Arrays with small, medium, and large beads. (A) Single beads are **(B)** separated by size, then by **(C)** dye concentration. **(D)** Medium and large beads are read based on PE-A concentration. **(E)** Raw PE-A Median values based on (D).

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® 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
GM-CSF	1500.00	500.00	166.67	55.56	18.52	6.17	2.06	0
IL-1β	6000.00	2000.00	666.67	222.22	74.07	24.69	8.23	0
IL-2	10000.00	3333.33	1111.11	370.37	123.46	41.15	13.72	0
IL-4	1000.00	333.33	111.11	37.04	12.35	4.12	1.37	0
IL-5	8330.00	2776.67	925.56	308.52	102.84	34.28	11.43	0
IL-6	1000.00	333.33	111.11	37.04	12.35	4.12	1.37	0
IL-10	2000.00	666.67	222.22	74.07	24.69	8.23	2.74	0
IL-13	1000.00	333.33	111.11	37.04	12.35	4.12	1.37	0
IL-15	11000.00	3666.67	1222.22	407.41	135.80	45.27	15.09	0
IL-22	100000.00	33333.33	11111.11	3703.70	1234.57	411.52	137.17	0
IL-17A	1000.00	333.33	111.11	37.04	12.35	4.12	1.37	0
IL-33	75000.00	25000.00	8333.33	2777.78	925.93	308.64	102.88	0
TNF-α	5555.56	1851.85	617.28	205.76	68.59	22.86	7.62	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.

	GM-CSF	IL-1β	IL-2	IL-4	IL-5	IL-6	IL-10	IL-13	IL-15	IL-17A	IL-22	IL-33	TNF-α
Std1 (pg/mL)	1500.00	6000.00	10000.00	1000.00	8330.00	1000.00	2000.00	1000.00	11000.00	1000.00	100000.00	75000.00	16666.67
Std1 MFI	9867.67	9275.67	8480.33	14256.33	11657.00	9636.33	13174.33	6409.33	9958.33	8954.33	4903.67	16275.00	10214.33
Std2 MFI	4892.33	7666.33	7200.00	9460.67	10364.67	3831.00	9265.00	4886.33	5377.00	5880.33	3741.00	9333.33	11049.33
Std3 MFI	3518.00	5645.33	5097.33	3954.33	7924.33	1715.67	5454.67	3176.33	2739.00	3258.33	2658.00	4623.33	9391.67
Std4 MFI	2020.33	3331.67	2906.00	1598.33	6007.33	739.00	2566.00	1715.33	1297.33	1646.33	1507.67	1882.67	6155.00
Std5 MFI	1688.00	1699.33	1424.33	914.33	4175.67	361.33	1046.33	851.33	587.00	822.00	776.33	618.67	3427.00
Std6 MFI	1063.33	820.67	689.33	659.00	2690.67	201.00	471.00	573.33	320.33	431.33	488.00	285.33	1792.67
Std7 MFI	935.67	402.33	350.67	582.00	1562.67	132.67	226.00	384.67	188.67	269.00	326.00	174.67	956.33
Negative Control MFI	1269.22	174.11	190.89	662.22	540.56	148.44	132.22	435.89	192.67	230.89	334.00	207.11	412.44

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)
GM-CSF	42.4
IL-1β	3.0
IL-2	7.5
IL-4	7.9
IL-5	1.0
IL-6	3.7
IL-10	1.3
IL-13	3.2
IL-15	19.7
IL-17A	0.9
IL-22	194.0
IL-33	189.1
TNF-α	14.1

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.

Target	Percent Recovery
GM-CSF	96.1%
IL-1β	102.8%
IL-2	103.1%
IL-4	101.7%
IL-5	106.7%
IL-6	97.0%
IL-10	100.0%
IL-13	100.0%
IL-15	96.7%
IL-17A	99.7%
IL-22	94.7%
IL-33	99.0%
TNF-α	100.0%

6. Linearity of Dilution

To test the linearity of dilution, samples (n = 9) were serially diluted in RayBio® Serum Diluent (Item 4) 1:3, 1:9, and 1:27. The measured concentrations of serially diluted samples were compared to that of the undiluted samples and averaged.

Target	Linearity of Dilution (%)
GM-CSF	105.3%
IL-1β	103.1%
IL-2	103.7%
IL-4	100.0%
IL-5	112.4%
IL-6	105.7%
IL-10	101.6%
IL-13	97.6%
IL-22	103.7%
IL-15	104.6%
IL-17A	100.8%
IL-33	102.0%
TNF-α	77.9%

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 #1, Sample #2). The intra-assay precision, or coefficient of variation (CV), of the samples is provided below.

Target	Sample #	Mean (pg/mL)	Standard Deviation	CV %
GM-CSF	1	169.61	13.13	7.74%
	2	52.12	4.31	8.27%
IL-1β	1	205.84	10.48	5.09%
	2	26.55	1.15	4.32%
IL-2	1	1112.09	35.71	3.21%
	2	123.13	4.17	3.39%
IL-4	1	112.83	3.56	3.16%
	2	8.58	0.57	6.58%
IL-5	1	103.85	5.10	4.91%
	2	32.93	0.32	0.98%
IL-6	1	118.81	2.72	2.29%
	2	38.72	0.92	2.37%
IL-10	1	248.32	3.60	1.45%
	2	21.76	1.00	4.58%
IL-13	1	144.79	4.71	3.25%
	2	9.36	0.55	5.93%
IL-15	1	1258.20	41.51	3.30%
	2	414.02	12.44	3.00%
IL-17A	1	130.69	10.35	7.92%
	2	9.35	0.47	4.98%
IL-22	1	11710.87	661.74	5.65%
	2	3591.91	217.91	6.07%
IL-33	1	8580.14	335.21	3.91%
	2	2812.30	118.81	4.22%
TNF-α	1	619.61	11.93	1.93%
	2	206.23	11.27	5.46%

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 #1, Sample #2). The inter-assay precision in (CV) of the samples is provided below.

Target	Sample #	Mean (pg/mL)	Standard Deviation	CV %
GM-CSF	1	416.81	17.43	4.18%
	2	60.58	2.32	3.84%
IL-1β	1	212.06	8.80	4.15%
	2	71.89	2.49	3.47%
IL-2	1	3410.66	33.94	1.00%
	2	119.64	4.93	4.12%
IL-4	1	342.27	7.46	2.18%
	2	111.77	1.51	1.35%
IL-5	1	306.71	10.56	3.44%
	2	107.26	4.83	4.50%
IL-6	1	317.75	10.08	3.17%
	2	125.54	9.51	7.58%
IL-10	1	71.79	2.41	3.36%
	2	22.98	1.72	7.47%
IL-13	1	338.64	38.02	11.23%
	2	35.61	1.99	5.59%
IL-15	1	3485.62	201.97	5.79%
	2	124.24	5.20	4.18%
IL-17A	1	329.82	9.01	2.73%
	2	35.74	2.48	6.94%
IL-22	1	3757.72	234.49	6.24%
	2	1094.76	101.74	9.29%
IL-33	1	9127.03	773.42	8.47%
	2	2698.95	160.30	5.94%
TNF-α	1	613.82	8.20	1.34%
	2	203.13	4.37	2.15%

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
Weak Signal	Low protein content in sample	Increase the length of the sample-bead incubation to overnight at 4°C (see Section D, step 3).
		Decrease sample dilution.
		Note that even if the incubation is increased or the sample dilution is decreased, it is not guaranteed that the protein will be detected.
	Detection antibody over diluted	Increase detection antibody concentration.
	Sample matrix effect	Optimize sample dilution.
		For serum samples, prepare the standards in the provided RayBio® Serum Diluent (Item 4).
		Adjust the PE MFI using a small amount of beads from the negative control to around $10^{2\sim3}$.
	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.
Poor standard curve	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 property diluted	Reconstitute the Lyophilized Protein Standard Mix (Item 5) on ice *before* making serial dilutions.
High background	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 $10^{2\sim3}$.
	Insufficient washing	Increase wash time. 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).
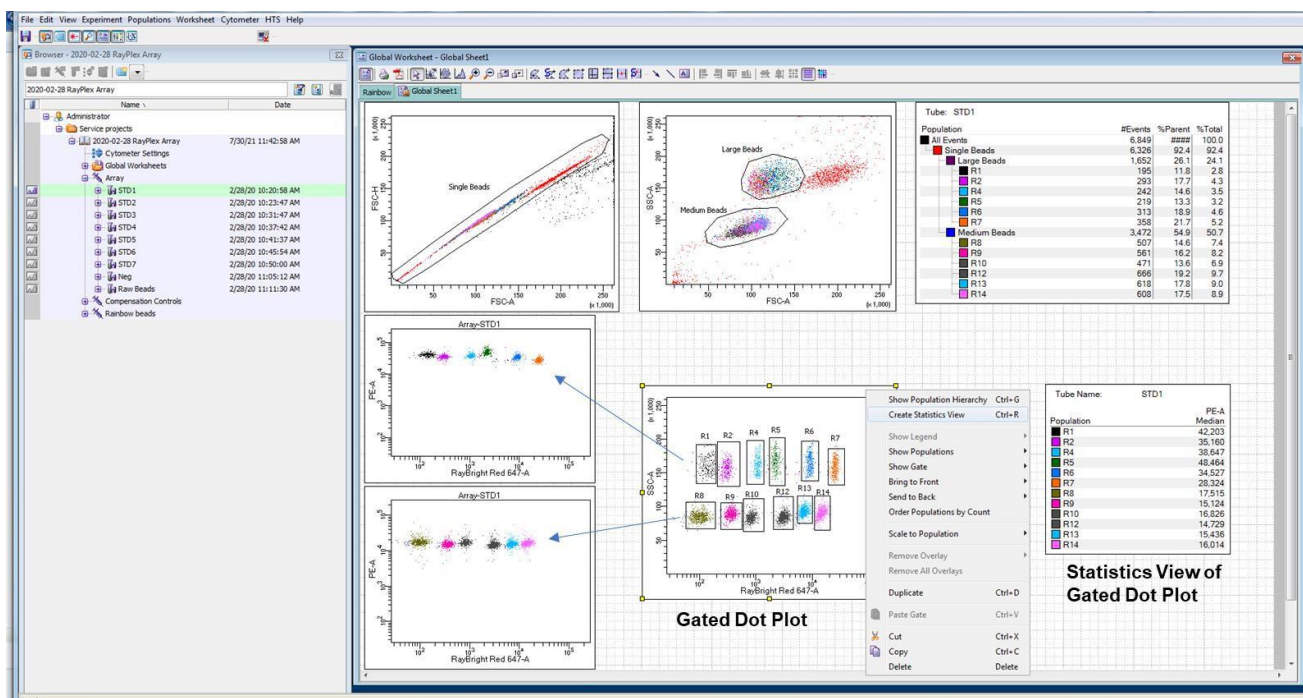

Figure 4. Screenshot of Creating Statistics View from the dot plot of gated beads populations. This is representative data only.

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", R1~R14). 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.
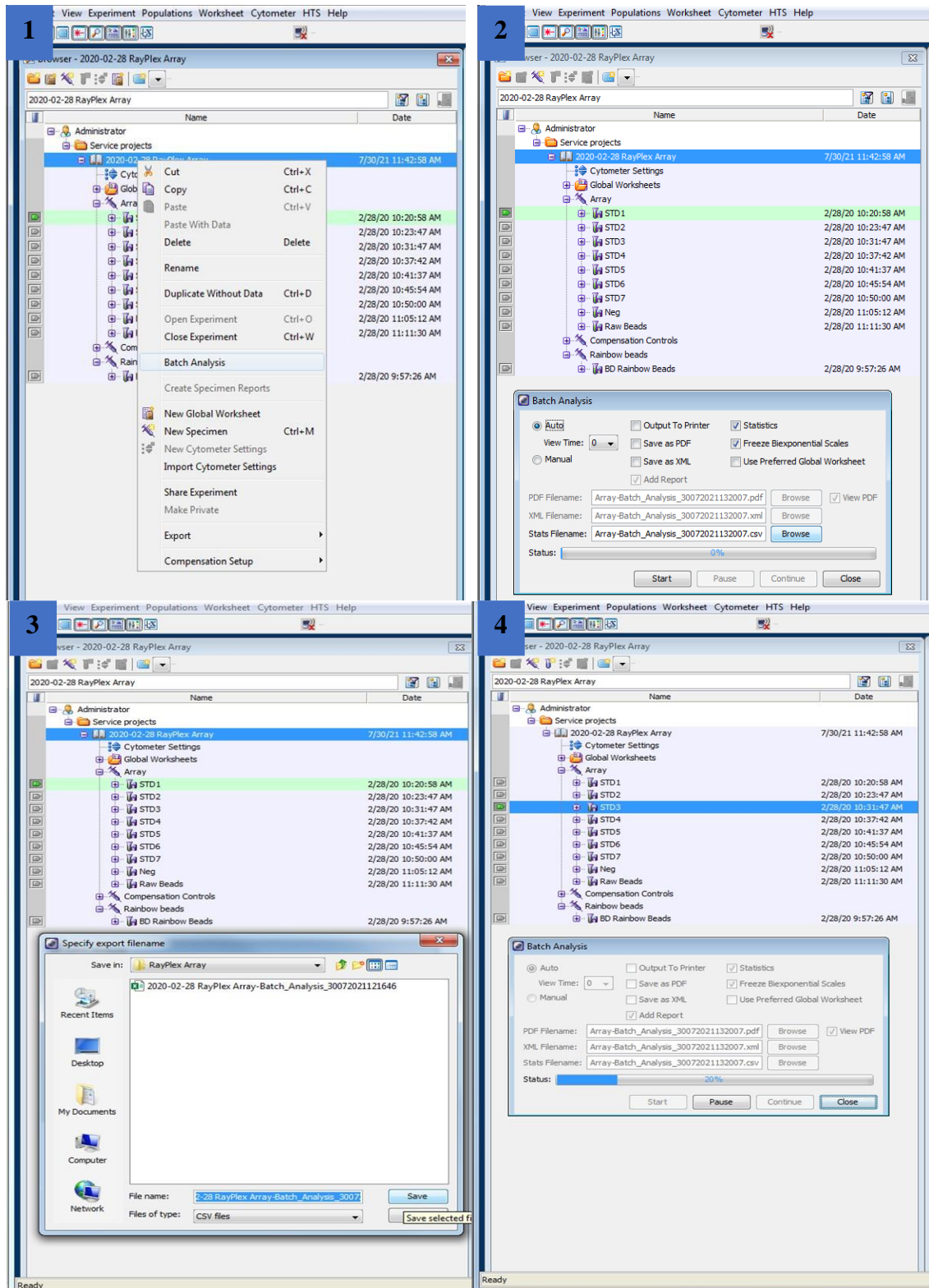
Figure 5. Screen shot showing how to begin the batch analysis through manual tube mode.
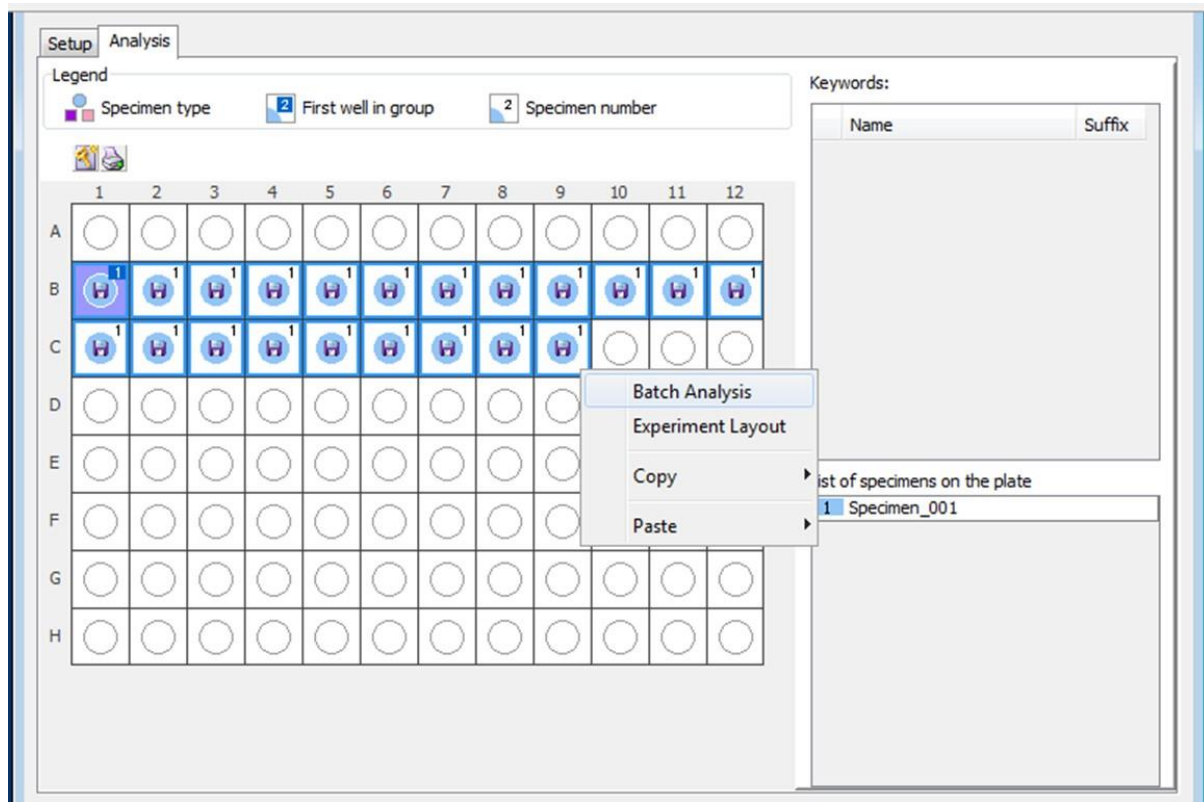
Figure 6. Screen shot showing how to begin HTS batch analysis.

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.
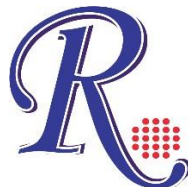
X.   Notes

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