

RayPlex[®] Anti-Drug Antibody Assay Kit

Quantitative Measurement of Anti-Drug Antibody by Flow
Cytometry

Catalog numbers:

ADA-PRELIM-50 (50 tests)

ADA-PRELIM-100 (100 tests)

ADA-PRELIM-200 (200 tests)

ADA-PRELIM-500 (500 tests)

User Manual Version 1.0

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I. Overview

Species	Human
ADAs Detected	Anti-Drug Antibody
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. <i>The optimal dilution must be determined empirically by the researcher.</i>
Number of Replicates	At least 2 replicates. Each replicate is considered a test.
Reproducibility	See Section VII (page 11) Assay Information for inter-CV and intra-CV percentages.
Assay Duration	4 hours

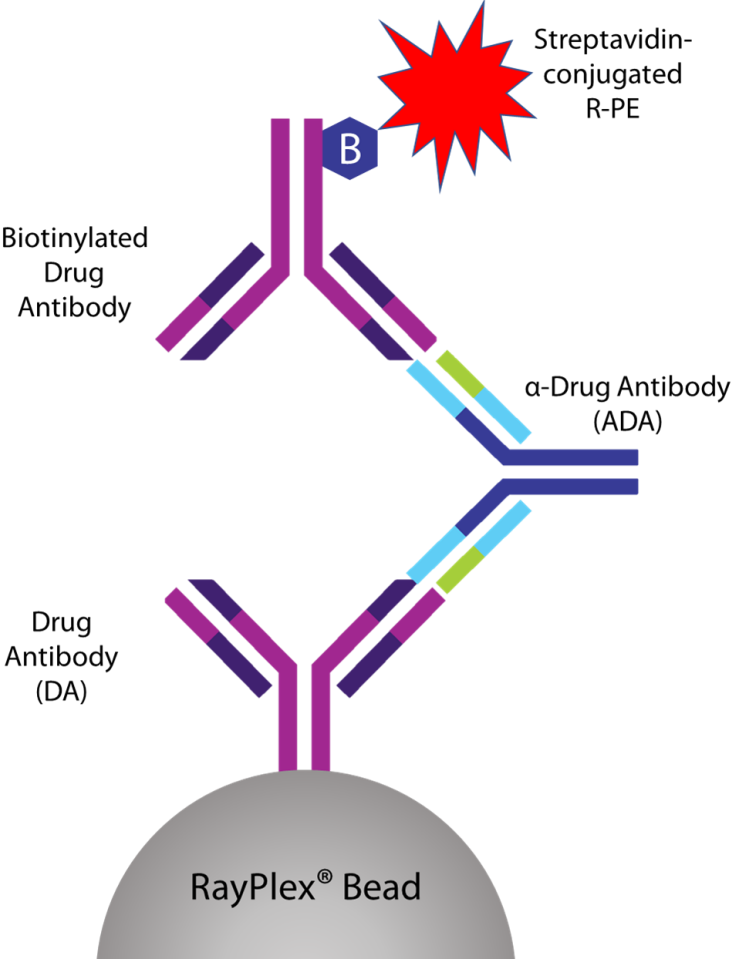
II. Introduction

Therapeutic agents, including antibodies and proteins, have been widely used to treat cancer, inflammation, allergy, and other diseases. However, host immune responses can significantly impact their pharmacokinetics, pharmacodynamics, safety, and efficacy. Drug immunogenicity-caused reactions, especially the anti-drug antibody (ADA) response, is the most measurable indicator of long-term efficacy of the treatment. Screening assays of ADAs are used to detect antibodies that bind to the therapeutic agent. The RayPlex[®] ADA Bead Assay is a sandwich- and bead-based fully quantitative assay for the detection of one or multiple ADAs using small sample volumes. It features high sensitivity, high stability, and high throughput with the reliable flow cytometry methodology. RayPlex[®] ADA Bead Assay requires no dedicated instrument and can be performed on most standard flow cytometers equipped with blue (PE) and red (APC) lasers.

III. How it Works

RayPlex[®] ADA Assays are tested as antibody bridge model. First drug antibody immobilized onto microbeads. After incubating the sample with the microbeads, ADAs are captured on the bead surface, and biotinylated same drug antibodies is added, followed by PE-conjugated streptavidin. This enables protein detection via the PE-streptavidin-biotin-antibody complex. In a multiplex setting, individual ADAs are

identified by their specific bead-fluorochrome combination, while the level of PE fluorescence reflects the amount of ADA that has been captured to the beads. The ADA level can be assessed (i.e., quantified) by comparing the PE signal to a standard curve generated from ADA standards at known concentrations.



A schematic showing how RayPlex[®] platform detects Anti-drug Antibody with flow cytometry.

IV. Bead ID and Distribution

1. General View of RayPlex MultiPlex Beads.

RayBiotech has three different sizes of RayPlex® Beads: small, medium, and large, (Figures 1 and 2).

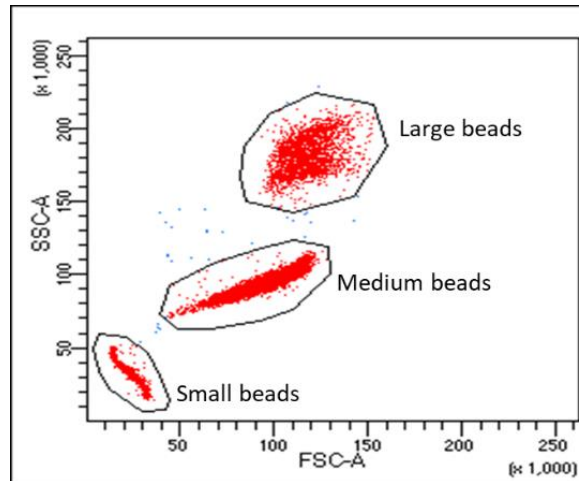


Figure 1. RayPlex® uses different size beads

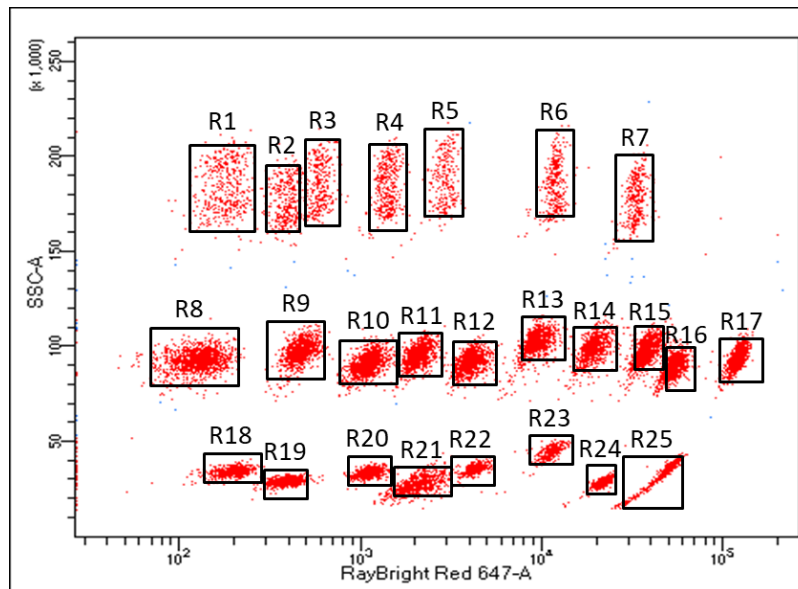


Figure 2. RayPlex® bead size and fluorescent intensities. *Bead ID* R1 - R7: Large Beads. *Bead ID* R8 - R17: Medium Beads. *Bead ID* R18 - R25: Small Beads.

2. Bead ID and associated target proteins in this kit.

This RayPlex® ADA Assay Kit uses the large bead population seen in Fig 1, specifically the R1 population in Fig 2.

V. Materials Provided

Upon receipt, all components should be stored at 4°C. The Lyophilized ADA Standard Mix (Item 6) should be stored at 4°C or below. *Use reagents immediately after preparation.* Additional vials of the Lyophilized ADA Standard Mix (Item 6) can be purchased separately. If stored at the proper temperature, the kit will retain complete activity for at least 6 months.

Item	Description	50 Tests	Per 100 Tests
1	Drug Antibody Conjugated-RayPlex® Beads	1.25 ml x 1 vial	1.25 ml x 2 vials
2	1X Assay Diluent	25 ml x 1 vial	25 ml x 2 vials
3	20X Wash Buffer	5 ml x 1 vial	5 ml x 2 vials
4	Pooled Normal Human Serum	0.5 ml x 1 vial	0.5 ml x 2 vials
5	Pooled Normal Human Plasma	0.5 ml x 1 vial	0.5 ml x 2 vials
6	Lyophilized ADA Standard	1	1
7	10X Biotinylated Drug Antibody	250 µl x 1 vial	250 µl x 2 vials
8	100X Streptavidin-PE	25 µl x 1 vial	25 µl x 2 vials
9	Flow Cytometer Setup Beads**	100 µl x 1 vial	100 µl x 1 vial
10	V-bottom 96-well Microplate	1	1
11	Filter 96-well Microplate	1	1
12	Manual	1	1

Notes:

- **Flow Cytometry Setup Beads (Item 9) is an additional smaller aliquot of Item 1 provided to assist in the setup of the flow cytometer.
- RayBiotech recommends using a Filter 96-well Microplate (Item 11) in lieu of the V-bottom 96-well microplate (Item 10) 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
- If using the Filter 96-Well Microplate (Item 11): Vacuum manifold compatible with a 96-well microplate
- 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 µl of diluted serum or plasma.
- We recommend analyzing samples in duplicate.
- Serum or Plasma Samples: Dilute serum or plasma samples with PBS at a dilution factor determined by end user.
- 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.

Note: *If you experience high background or the readings exceed the detection range, further dilution of your sample may be recommended.*

B. Preparation of Reagents

- Keep all reagents on ice.
 - Protect fluorescent RayPlex® Beads from light.
1. Vortex the RayPlex® Bead (Item 1) for 30 seconds before use. Use 25 µl beads per test.
 2. Dilute the 20X Wash Buffer (Item 3) 20-fold with de-ionized (DI) H₂O to prepare 1X Wash buffer. Dilute only what is needed to perform the tests for each experiment. (See Example note below.)
 3. Dilute the 10X Biotinylated Drug Antibody (Item 7) 10-fold in 1X Assay Diluent (Item 2) to prepare a 1X Biotinylated Drug Antibody working stock. Dilute only what is needed to perform the tests for each experiment. Use 50 µl per test.
 4. Dilute the 100X Streptavidin-PE (Item 8) 100-fold in 1X Assay Diluent (Item 2) to prepare a 1X Streptavidin-PE working stock. Dilute only what is needed to perform the tests for each experiment. Use 50 µl per test.

Example: A 20-fold dilution would be 1-part 20X Wash Buffer and 19 parts DI H₂O.

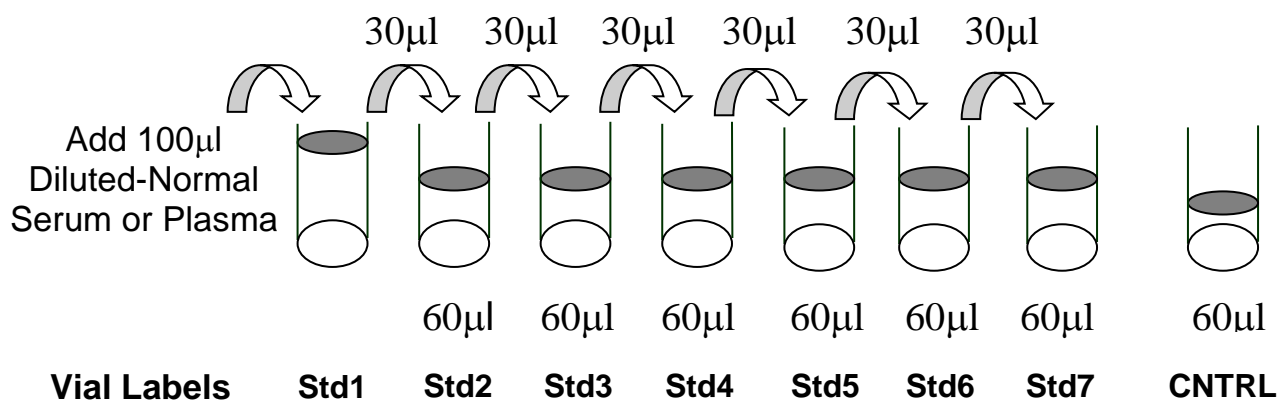
C. Preparation of ADA Standards

1. Spin the Lyophilized ADA Standard Mix (Item 6) briefly (1 – 3 seconds) to collect contents to the bottom of the vial.
2. Depending on whether the tested samples are serum or plasma, reconstitute the Lyophilized ADA Standard Mix (Item 6) by adding 100 μ l diluted normal human serum or plasma (Item 3 or 4; diluted with PBS at the same dilution factor as serum or plasma samples) to the tube. Dissolve the powder thoroughly and gently by pipetting up and down 5 – 10 times. Label this tube as Std1.

Note: Reconstitute the Lyophilized ADA Standard Mix (Item 6) within one hour of usage.

3. Obtain 6 clean 1.5 ml microcentrifuge tubes (or use a 96-well round-bottom plate). Label the tubes as Std2 to Std7. Add 60 μ l diluted normal human serum or plasma (as above) to each tube.
4. Pipette 30 μ l Std1 into tube “Std2” and mix gently. Perform 5 more serial dilutions by adding 30 μ l Std2 to tube “Std3,” mix, and so on. (See also image below)

Prepare serial dilutions of standards



5. Add 60 μ l 1X Assay Diluent (Item 2) 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: 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 10) or 1.2 μm Filter 96-well Microplate (Item 11) 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 11) in lieu of the V-bottom 96-well microplate (Item 10) 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[®] Beads (Item 1) to each well that will contain the CNTRL, Standard, or Sample.
3. Add 25 μl of the CNTRL, Standard, or Sample to the appropriate well. The total volume in each well is now 50 μl . 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).
 - **V-bottom 96-well Microplate (Item 10):** 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 11):** 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 50 μl of 1X Biotinylated Drug Antibody (*diluted* Item 7) (see Section B, Preparation of Reagents) 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 μ l of 1X Streptavidin-PE (*diluted* Item 7) (see Section B, Preparation of Reagents) 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 μ l of 1X Wash Buffer (*diluted* Item 3).
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 10)
 - 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[®] Drug Antibody Anti-Drug Antibody Assay Kit requires a flow cytometer 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.

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 Cytometry Setup Beads (Item 9) in 200 μ 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) Then gate on the “Large bead” population (R1 large bead is used in this kit) (Figure 3B).
6. Create a new dot plot from parent gate in step 5 Figure 3B. Gate on R1 populations for the assay based on SSC (linear mode) and APC (log mode). (Figure 3C).
7. Create a dot plot of APC versus PE (using log scale for both) from the R1 population. Run the Flow Cytometry Setup Beads (Item 9). Adjust the PE voltage so that the PE mean fluorescent intensity (MFI) is around $10^2\text{--}3$.
8. Set the number of bead events to be acquired to at least 300 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 of dot plot with R1 population (Figure 3C) to show the PE MFI for R1 population (Figure 3E).

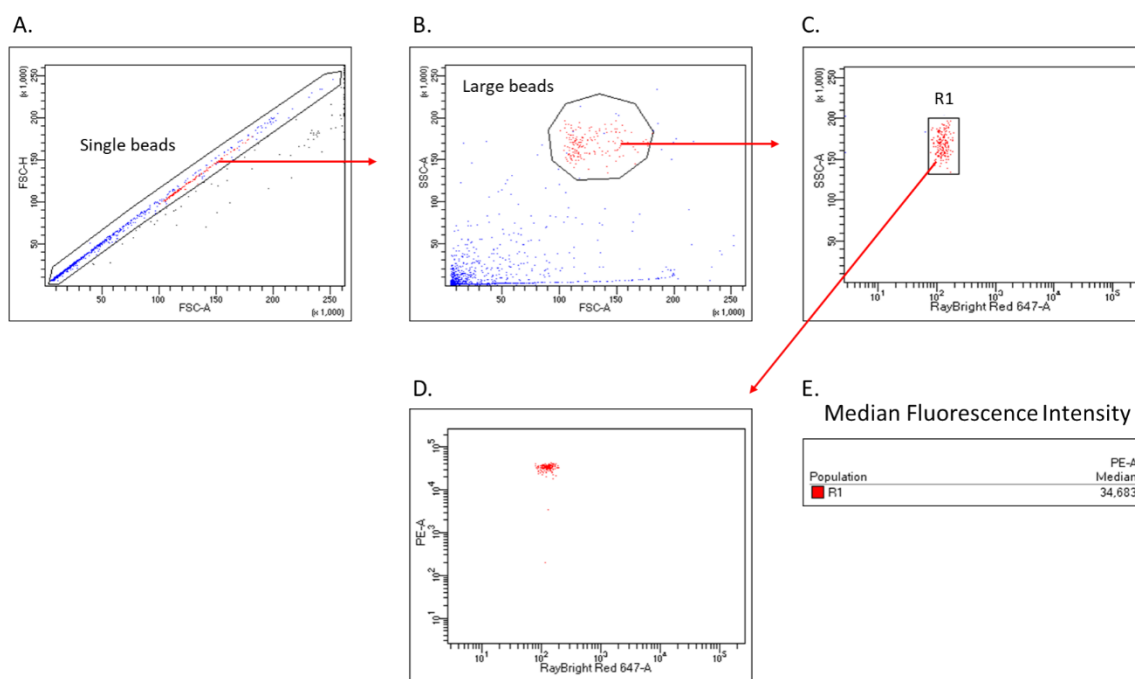


Figure 3. Representative Images of Gated Flow Cytometry Scatter Plots Using RayPlex® Arrays. (A) Single beads; (B) Large beads; (C) R1 beads; (D) Sample reading based on PE-A concentration; (E) Statistic view of PE-A MFI value for R1 population.

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 Quality 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.

2. Representation of Standard Curve MFI Values

The figure shows a representative 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. Five-parameter Logistic Curve Fitting will be applied for regression and calculations.

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

The LOD will be determined during product development process.

Note: The LOD may vary run-to-run and should be determined empirically.

4. Percent (%) Recovery of Spiked Standards

The recovery rate in Pooled Normal Human Serum will be determined during development process. For spike recovery of standards, target ADA standard with concentrations corresponding to standard 2 (Std2) and standard 4 (Std4) will be spiked into 10% Pooled Human Serum (Item 4) across 4 replicates. The spiked samples will be assayed, and the measured concentrations will be compared to the expected concentrations.

5. Linearity of Dilution

The linearity of Dilution in Pooled Normal Human Serum will be determined during development process. To test the linearity of dilution, ADA will be reconstituted in 10% Pooled Normal Human Serum (Item 4) as “Undiluted Sample”. Triplicate undiluted samples (n = 3) will be serially diluted in 10% Pooled Normal Human Serum (Item 4) 1:3, 1:9, and 1:21. The measured concentrations of serially diluted samples will be compared to that of the undiluted samples and averaged.

6. Intra- and Inter-Assay Precision

Intra-Assay CV: < 10%

Inter-Assay CV: < 10%

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. <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. For serum samples, prepare the standards in the provided Pooled Normal Human Serum (Item 4). Adjust the PE MFI of the negative control using the Flow Cytometry Setup Beads (Item 9) 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.
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 6) according to manual.
	Lyophilized Protein Standard Mix (Item 5) degraded or not properly diluted	Reconstitute the Lyophilized Protein Standard Mix (Item 6) on ice <i>before</i> making serial dilutions.
High background	Improper flow cytometer setup and optimization	Run Flow Cytometry Setup Beads (Item 9) 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 so that the negative control MFI using the Flow Cytometry Setup Beads (Item 9) is around 10^{2-3} .
	Insufficient washing	Increase wash time. Use more wash buffer.

IX. Appendix

RayPlex® Data Analysis Using BD FACS Diva 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).

Note: The data in the following instruction is for demonstration only, they may not be related to a specific RayPlex® kit.

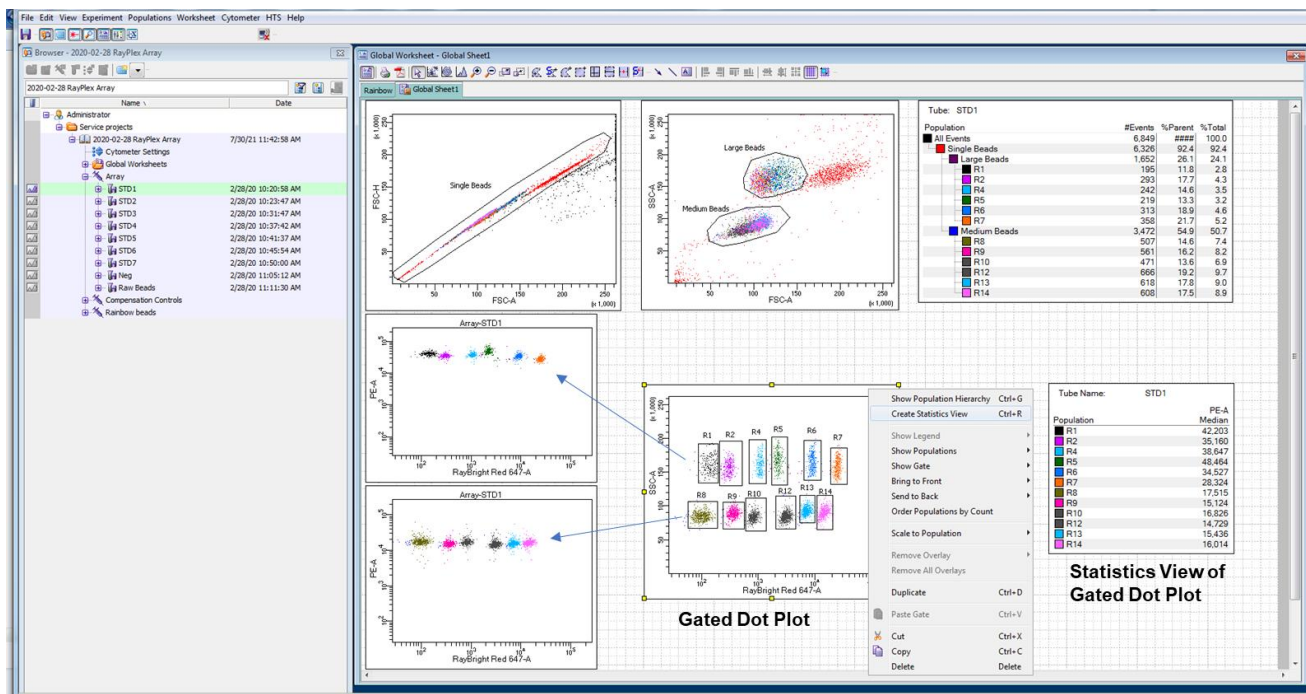
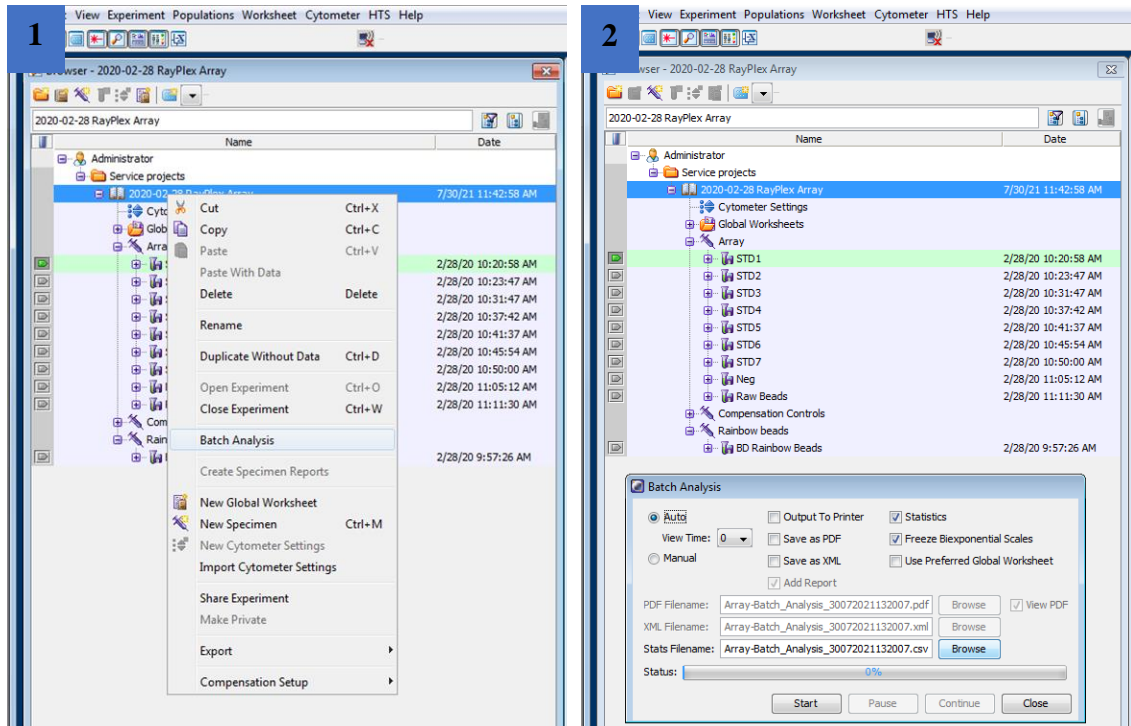


Figure 4. Screenshot of Creating Statistics View from the dot plot of gated beads populations.

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.



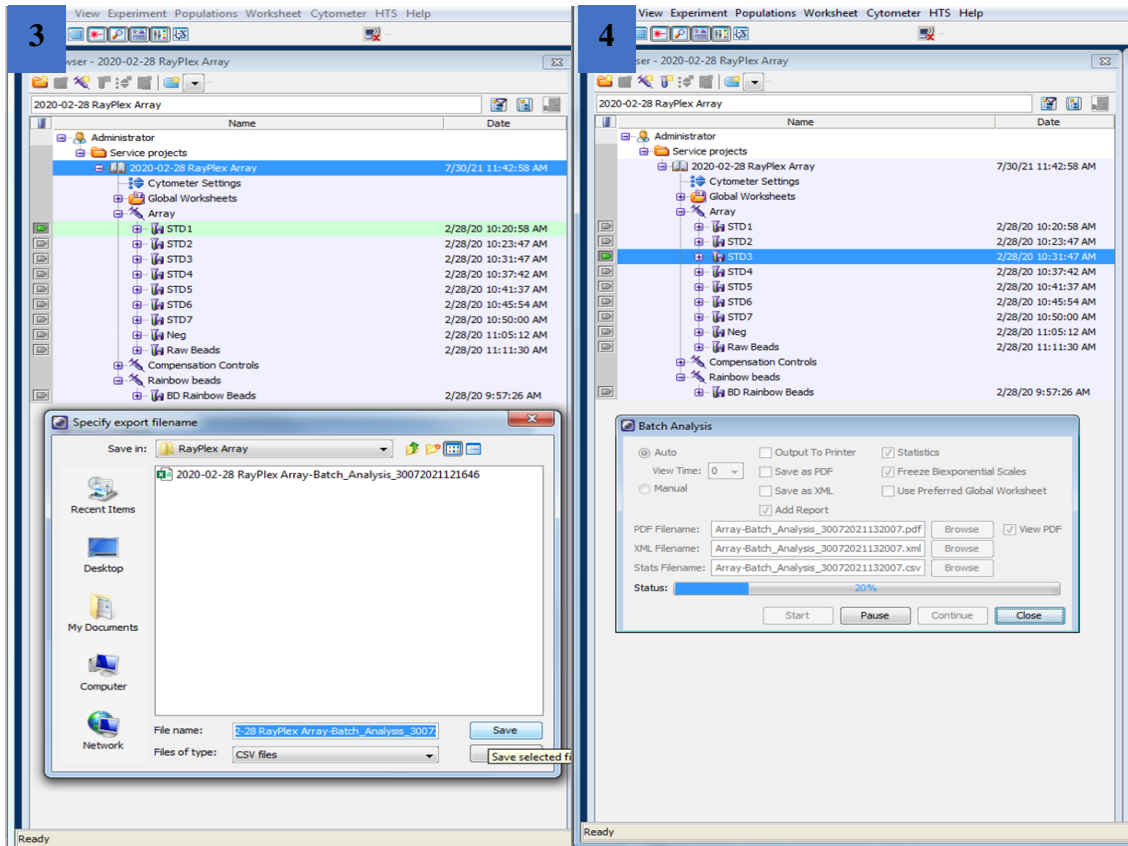


Figure 5. Screen shot showing how to begin the batch analysis through manual tube mode.

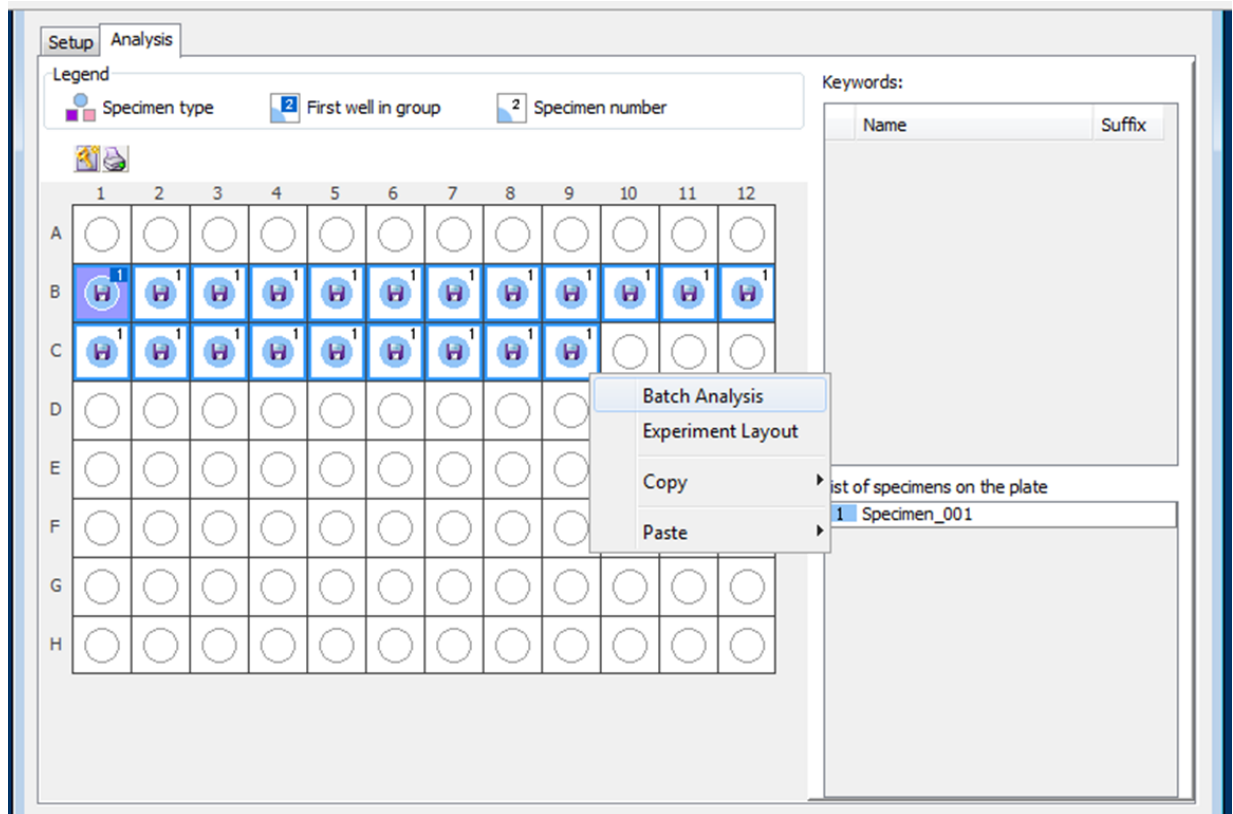


Figure 6. Screen shot showing how to begin HTS batch analysis.

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).

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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