RayPlex®
Anti-Drug Antibody Assay Kit

Quantitative Measurement of Anti-Drug Antibody (ADA) by Flow Cytometry

Catalog numbers:
ADA-X-48 (48 tests)
ADA-X-96 (96 tests)
ADA-X-192 (192 tests)
ADA-X-480 (480 tests)

User Manual Version 5.0
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RayBiotech
Empowering your proteomics

ISO 13485 Certified

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I. Overview

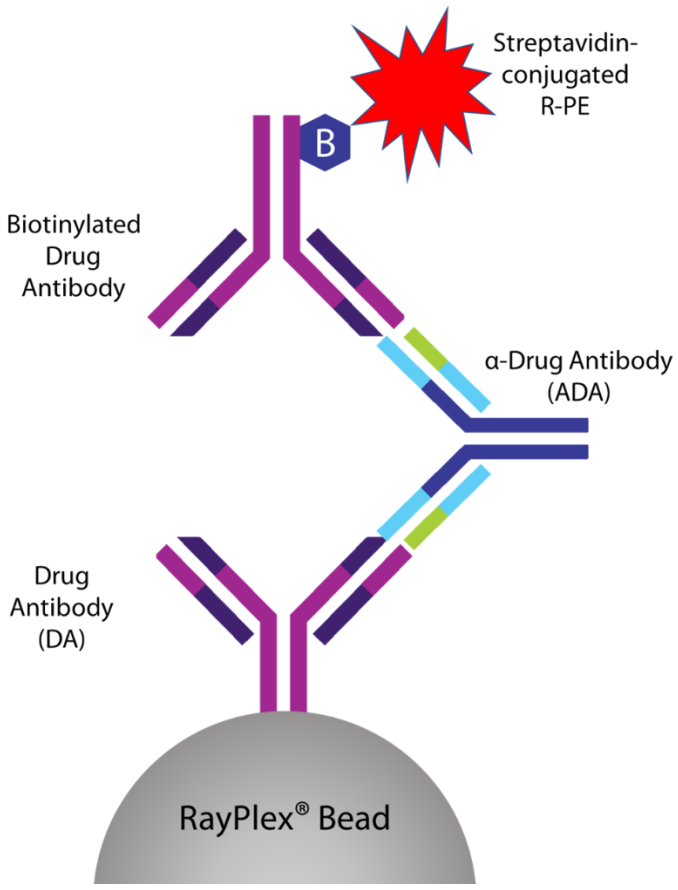
Species	Human
ADAs Detected (1)	Anti-X Antibody
Format	Bead-based
Detection Method	Flow Cytometry using a flow cytometer equipped with a blue (or green) laser (PE channel) and red laser (APC channel)
Minimal Sample Volume	25 µL per test after dilution. *The optimal dilution must be determined empirically by the researcher.*
Number of Replicates	At least 2 replicates. Each replicate is considered a test.
Reproducibility	See Section VII (page 15) 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, are 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 or green (PE) and red (APC) lasers.

III. How it Works

RayPlex® ADA Assays are tested using the antibody bridge model. First, the drug antibody is immobilized onto microbeads. After incubating the sample with the microbeads, ADAs are captured on the bead surface, and biotinylated same drug antibodies are 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

RayBiotech has three different sizes of RayPlex® Beads: small, medium, and large, (Figures 1 and 2). This RayPlex® ADA Kit uses the **X** bead population seen in Fig 1, specifically the **RX** population in Fig 2.

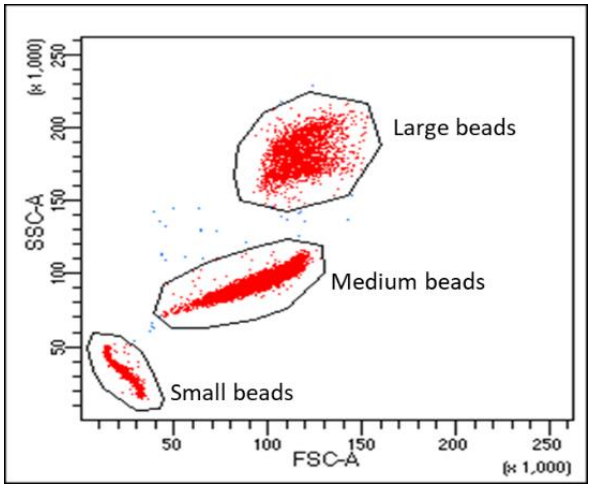


Figure 1. RayPlex® uses different size beads

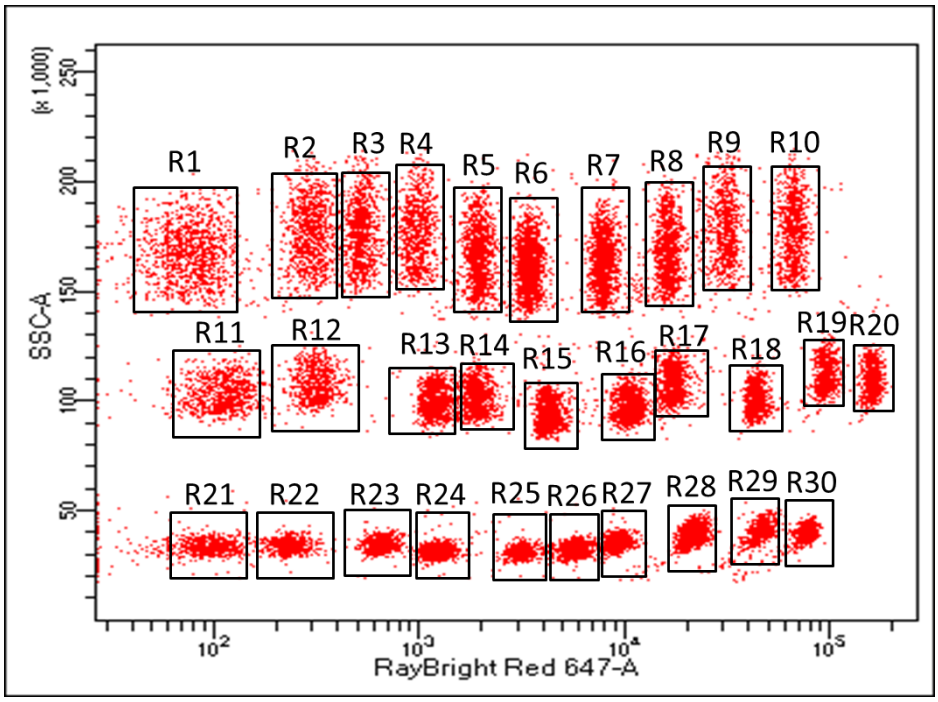


Figure 2. Different populations of bead size and color combinations ("R#") enable multiplex protein detection with RayPlex® arrays. *Bead ID* R1 – R10: Large Beads. *Bead ID* R11 – R20: Medium Beads. *Bead ID* R21 – R30: Small Beads.

V. Materials Provided

Upon receipt, the Liquid ADA 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 Liquid ADA Standard Mix (Item 5) can be purchased separately (cat no. ADA-X-Item5). If stored at the proper temperature, the kit will retain complete activity for at least 6 months.

Catalog #	Description	Per 48 Tests	Per 96 Tests
ADA-X-Item1	DA Conjugated-RayPlex® Beads	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
ADA-X-Item5	Liquid ADA Standard	10 µL x 1 vial	10 µL x ***2*** vials
ADA-X-Item6	1X Biotinylated DA	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
ADA-X-Item8	Flow Cytometer Setup Beads**	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 Beads (Item 8) are provided to set up the flow cytometer.*
2. ***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 coefficients of variation (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 (or green) and red lasers capable of measuring phycoerythrin (PE) and allophycocyanin (APC) channels
- Aluminum foil or 96-well Plate Aluminum Sealers
- Deionized 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 µL of diluted serum or plasma, cell culture media, or other fluids.
- Optimal sample dilutions will need to be determined by each researcher empirically, but a **1000-fold dilution with 1X Assay Diluent (Item 2) or PBS for serum/plasma** may be used as a starting point.
- 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.

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® Beads (Item 1) for 30 seconds before use. Use 25 µL beads per test.
3. Dilute the 20X Wash Buffer (Item 3) 20-fold with deionized (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)

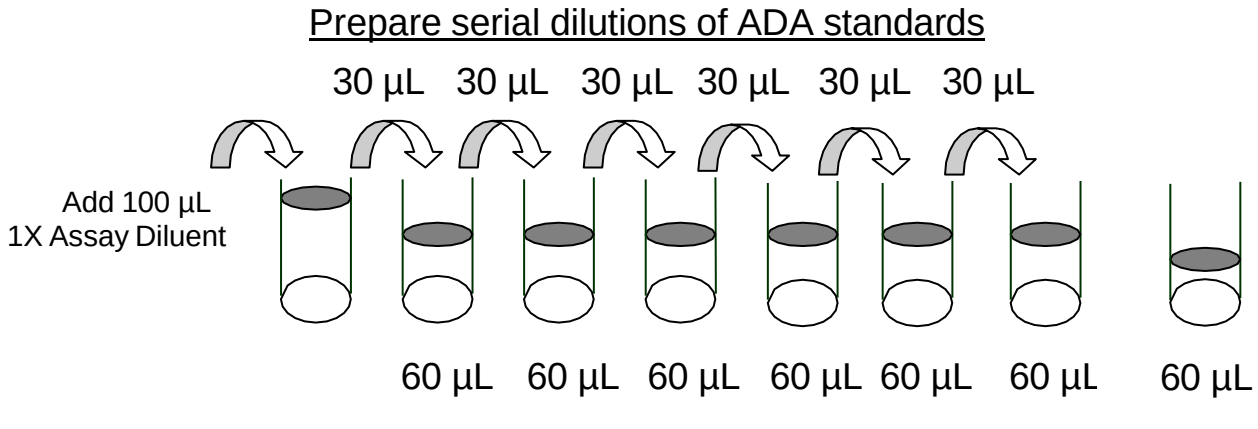
to prepare a 1X Streptavidin-PE working stock. Use 50 µL per test.

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

C. Preparation of ADA Standards

1. Centrifuge the Liquid ADA Standard (Item 5) briefly (1-3 seconds) to collect contents to the bottle of the vial.
2. Reconstitute the Liquid ADA Standard (Item 5): Add 100 µL 1X Assay Diluent (Item 2) to the tube. Dissolve the Standard thoroughly and gently by pipetting up and down 5-10 times. Label this tube as Std1.

   Note: *Use the Liquid ADA Standard (Item 5) within 1 hour of preparation.*
3. Obtain six 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) as applicable to each tube.
4. Pipette 30 µL Std1 into tube Std2 and mix gently. Perform five more serial dilutions by adding 30 µL of Std2 to tube Std3, mix, and so on. (See image below)



5. Add 60 µL 1X Assay Diluent (Item 2) 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 1.2 µm 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 coefficients of variation (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 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. 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® ADA Assay requires a flow cytometer that is equipped with blue (or green) 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 Beads (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.

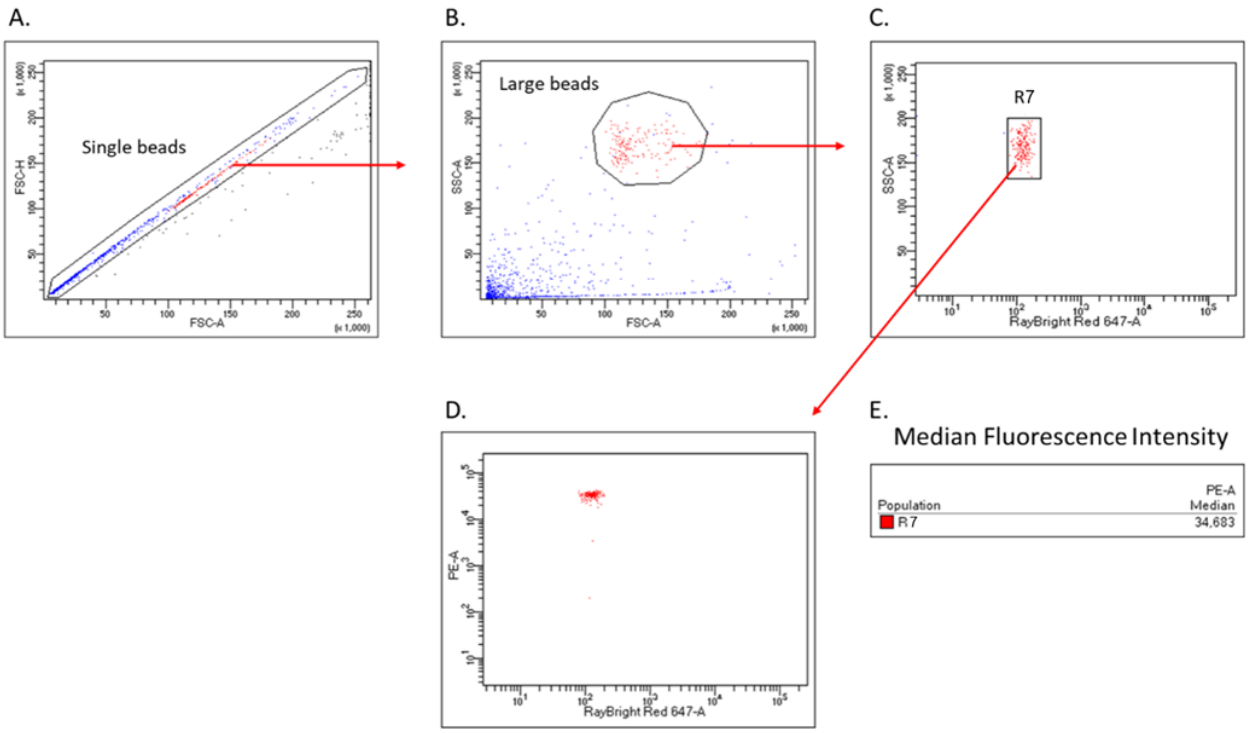


Figure 3. Representative Images of Gated Flow Cytometry Scatter Plots Using RayPlex® Arrays. (A) Single beads; **(B)** Medium beads; **(C)** R3 beads; **(D)** Sample reading based on PE-A concentration; **(E)** Statistic view of PE-A MFI value for R population (R# varies for different kits).

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 FACSDiva 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. Bead ID and associated Target Protein

RX: Anti-X

2. 8-Point Standard ADA Concentrations (pg/mL)

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

3. 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.

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

The LOD is the minimum detectable concentration (i.e., sensitivity) of the assay, which is based on a triplicate testing of 8-point standards. It was determined by spiking each ADA standard into 1X Assay Diluent (Item 2).

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

5. Percent (%) Recovery of Spiked Standards

For spike recovery of standards, target proteins with concentrations corresponding to standard 2 (Std2) were spiked into 1X Assay Diluent (Item 2) across 9 replicates. The spiked proteins were assayed, and the measured concentrations were compared to the expected concentrations.

6. Linearity of Dilution

To test the linearity of dilution, samples (n = 9) were serially diluted in 1X Assay Diluent (Item 2) 1:3, 1:9, and 1:27. The measured concentrations of serially diluted samples were compared to that of the undiluted samples and averaged.

7. Intra- and Inter-Assay Precision

1) Intra-Assay Precision

Spiked standard protein in 1X Assay Diluent (Item 2) 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.

2) Inter-Assay Precision

Spiked standard protein in 1X Assay Diluent (Item 2) was analyzed in triplicate across three independent assays. This was performed at two different concentrations per standard protein (i.e., Sample #2, Sample #3). The inter-assay precision in (CV) of the samples is provided below.

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. Adjust the PE MFI of the negative control using the Setup Beads (Item 8) 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.
	Liquid ADA Standard (Item 5) not properly prepared	Serially dilute the Liquid ADA Standard (Item 5) according to manual.
	Liquid ADA Standard (Item 5) degraded or not property diluted	Reconstitute the Liquid ADA Standard (Item 5) on ice before making serial dilutions.
High background	Improper flow cytometer setup and optimization	Run Setup Beads (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 FACSDiva. 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.

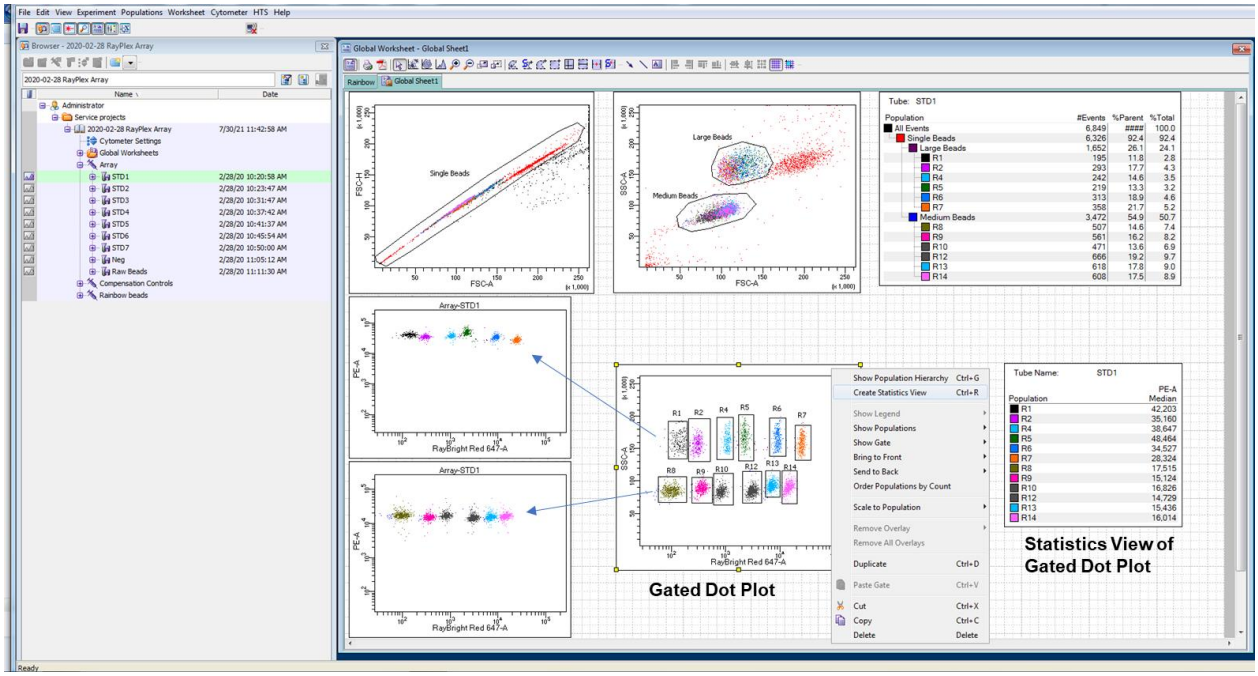


Figure 4. Screenshot of Creating Statistics View from the dot plot of gated beads populations. This is representative data only.

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.

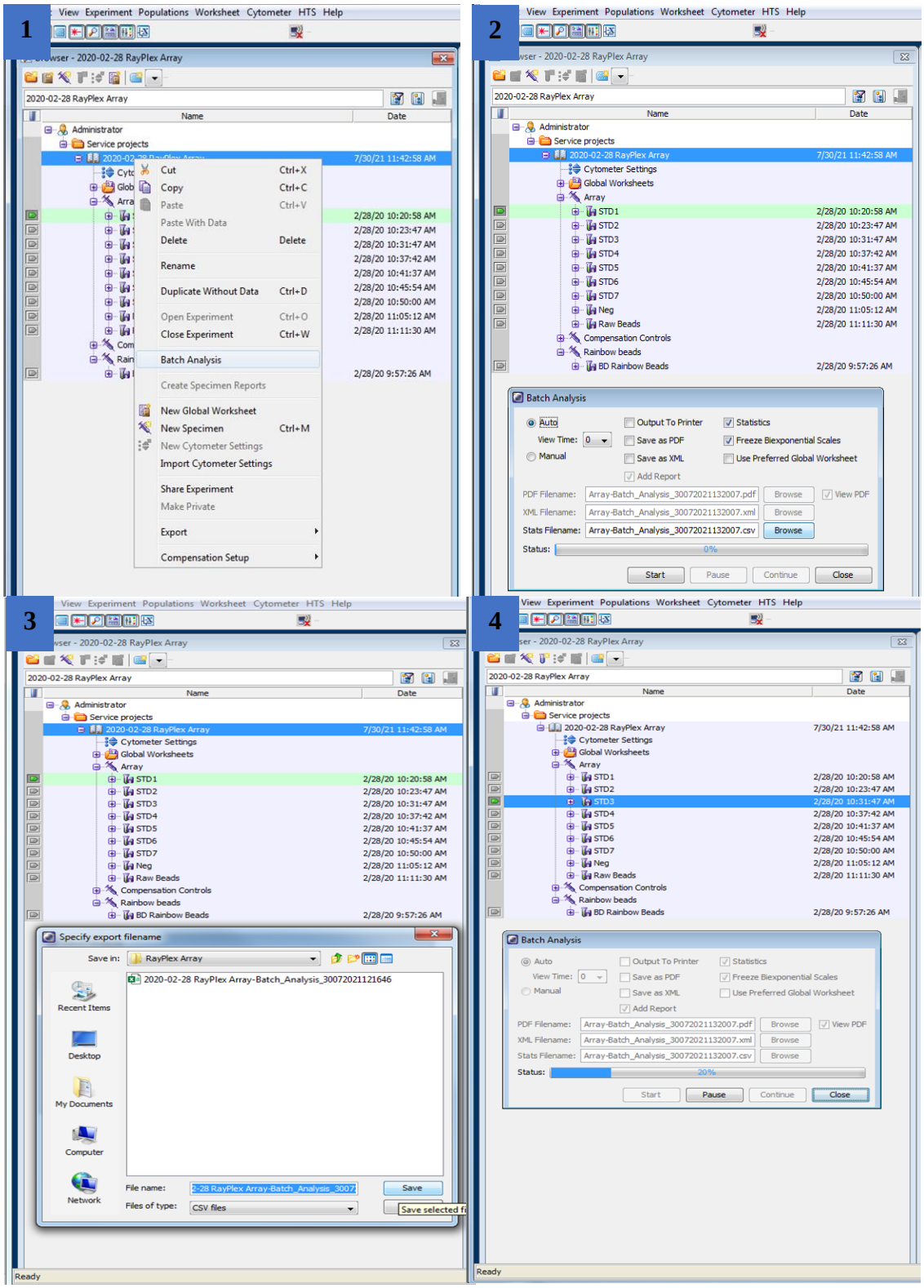


Figure 5. Screen shot showing how to begin the batch analysis through manual tube mode.

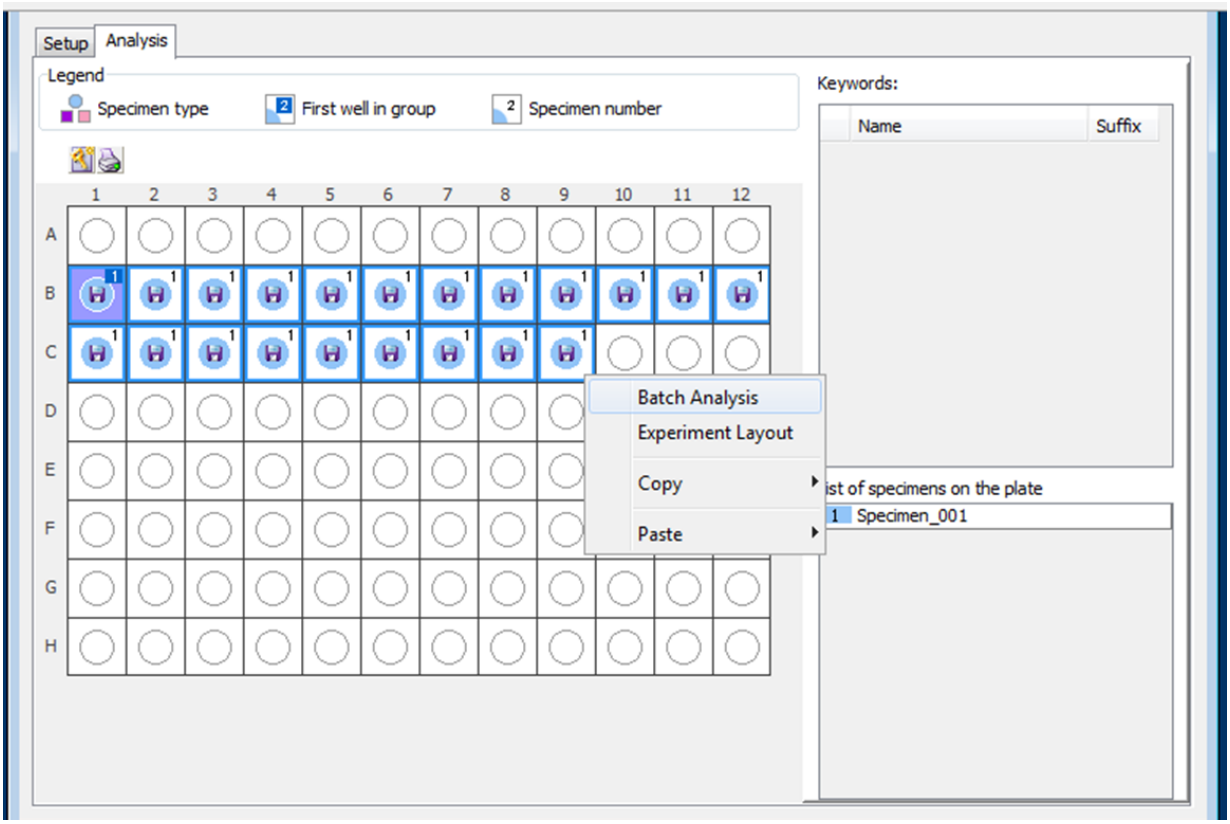
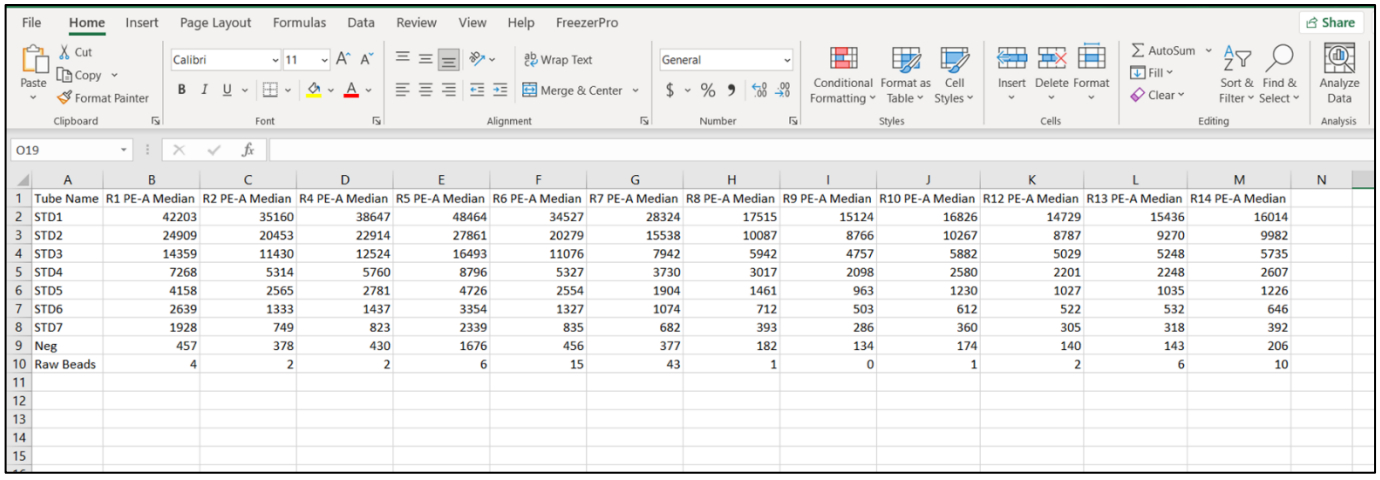


Figure 6. Screen shot showing how to begin HTS batch analysis.

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).



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
STD1	42203	35160	38647	48464	34527	28324	17515	15124	16826	14729	15436	16014
STD2	24909	20453	22914	27861	20279	15538	10087	8766	10267	8787	9270	9982
STD3	14359	11430	12524	16493	11076	7942	5942	4757	5882	5029	5248	5735
STD4	7268	5314	5760	8796	5327	3730	3017	2098	2580	2201	2248	2607
STD5	4158	2565	2781	4726	2554	1904	1461	963	1230	1027	1035	1226
STD6	2639	1333	1437	3354	1327	1074	712	503	612	522	532	646
STD7	1928	749	823	2339	835	682	393	286	360	305	318	392
Neg	457	378	430	1676	456	377	182	134	174	140	143	206
Raw Beads	4	2	2	6	15	43	1	0	1	2	6	10

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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