RayPlex® Human Alzheimer's Disease Magnetic Array 1

Quantitative Measurement of 2 Human Proteins
by Flow Cytometry

Catalog numbers:
FAHM-ALZ-1-48 (48 tests)
FAHM-ALZ -1-96 (96 tests)
FAHM-ALZ -1-192 (192 tests)
FAHM-ALZ -1-480 (480 tests)

User Manual Version 5.0

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RayBiotech
Empowering your proteomics

ISO 13485:2016

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Table of Contents

I. Overview

Species	Human
Proteins Detected (2)	pTau217 and pTau181
Format	Bead-based
Detection Method	Flow Cytometry using a Flow Cytometer equipped with a blue (or green) laser (PE channel) and red laser (Alexa Fluor 700 and Alexa Fluor 647/APC channel) OR Luminex instrument
Minimal Sample Volume	25 µL per test after dilution. *The optimal dilution mustbe determined empirically by the researcher*
Number of Replicates	At least 2 replicates. Each replicate is considered a test
Reproducibility	See Section VII (page 22) for inter-CV and intra-CV percentages for each protein
Assay Duration	4 hours

II. Introduction

Alzheimer's Disease is a neurodegenerative disease affecting one's memory and cognitive function. The accumulation of beta-amyloid and phosphorylated Tau has been identified as having a strong correlation to the disease. This kit detects two forms of phosphorylated Tau: pTau217 and pTau181. Both forms are associated with the early stages of disease, but the role they play in Alzheimer's Disease progression is not fully understood. Research focused on these targets can aid in the study of the disease and patient prognosis.

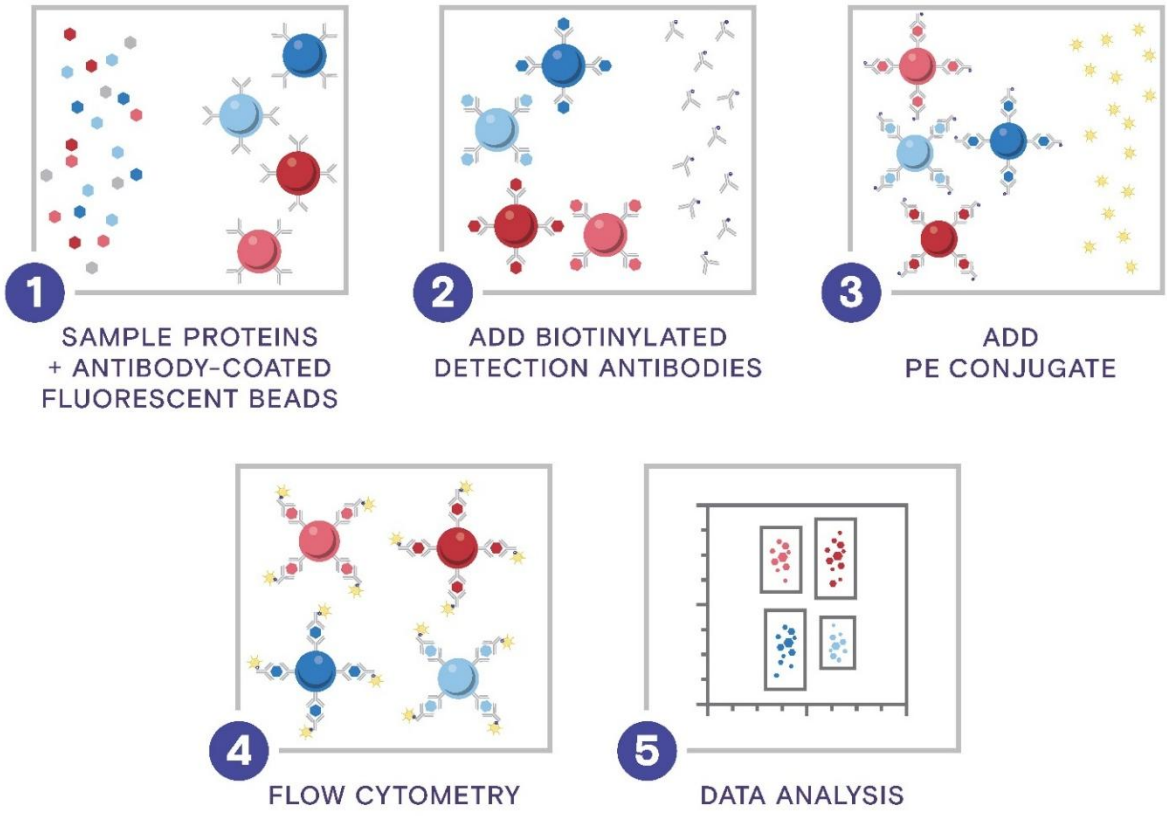
The traditional technique for detecting and quantifying proteins one at a time is the enzyme-linked immunosorbent assay (ELISA). However, using this method to detect multiple proteins – particularly for precious samples with small quantities – becomes challenging as the processing time and required sample 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 couples 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 common flow cytometers equipped with blue (or green) and red lasers. This kit can also be used with Luminex systems.

This kit uses magnetic beads that allow the use of an automatic plate washing system with magnetic separation ability during washing steps. For data in this manual, a BioTek 405 TS Washer and LifeSep™ 96F magnetic separator unit were used. Further details are included in Section VI.

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 Conjugate are added, thus enabling protein detection via the sandwich immunocomplex. 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.

*PE Conjugate consists of PE-conjugated Streptavidin or PE-conjugated Anti-Biotin depending on kit optimization.

IV. Bead ID and Distribution

1. General View of RayPlex® Multiplex Beads.

RayPlex® Human Alzheimer's Disease Magnetic Bead Array uses M-100 series magnetic multiplex beads which have 100 populations based on the barcode determined by the incorporated dual fluorochrome with different intensities. All the M-100 beads are equal in size. This kit uses 2 populations out of 100. (Figures 1 and 2).

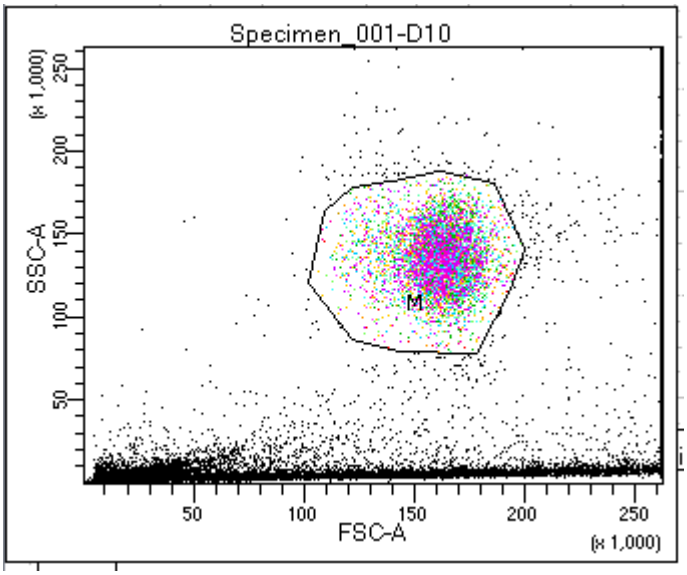


Figure 1. RayPlex® M100 beads are the same size.

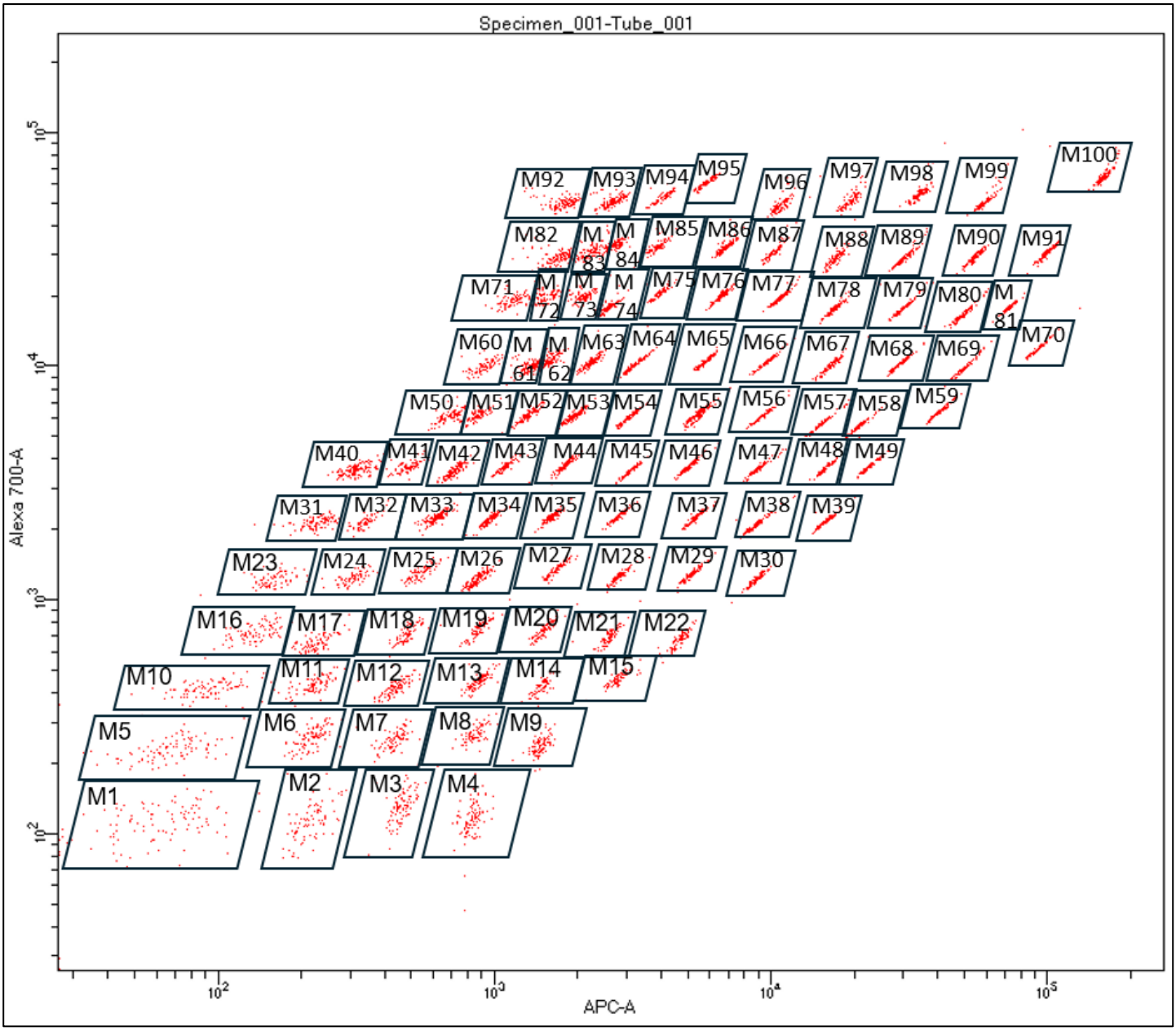
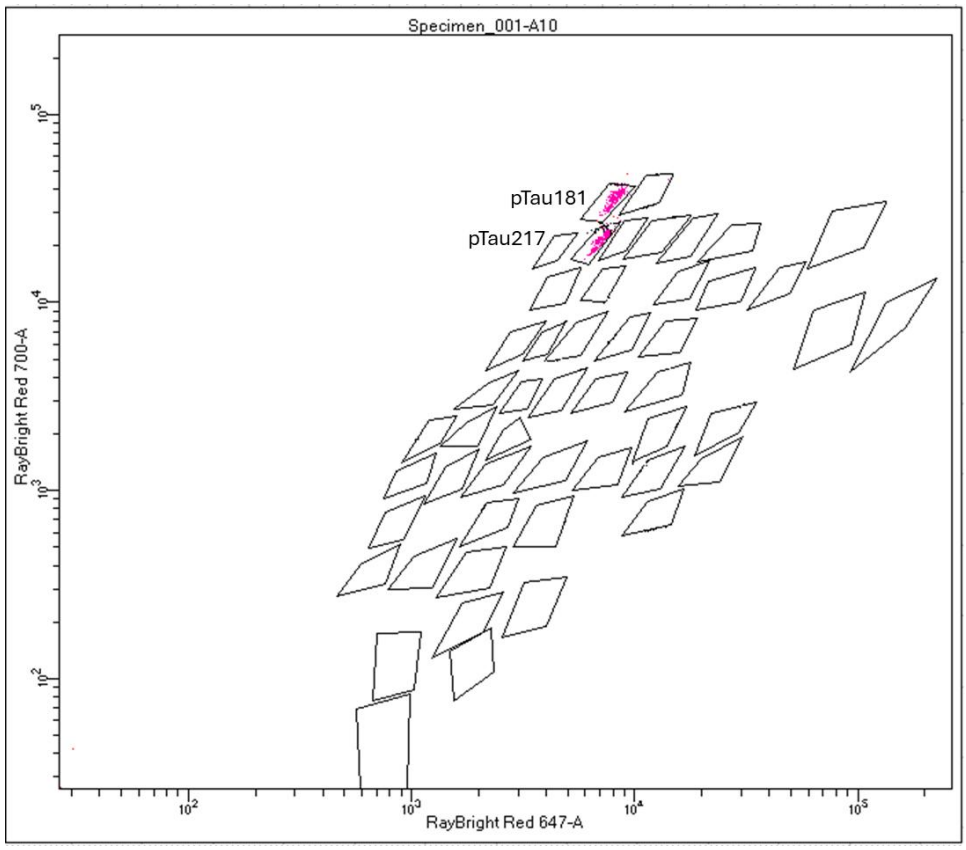


Figure 2. Different populations of beads with dual fluorescence combinations ("M#") enable multiplex protein detection with RayPlex® arrays.

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

Target	Bead ID
pTau217	M84
pTau181	M92



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 # FAHM-ALZ-1-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
FAHM-ALZ-1-Item1	RayPlex® Magnetic 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
137-00022-Item4	RayBio® Human Serum/Plasma Diluent	2.5 mL x 1 vial	2.5 mL x ***2*** vials
FAHM-ALZ-1-Item5	Lyophilized Protein Standard Mix	1	**2**
FAHM-ALZ-1-Item6	1X Biotinylated Detection AntibodyCocktail	1.25 mL x 1 vial	1.25 mL x ***2*** vials
FAX-PE-Item7	10X PE Conjugate	250 µL x 1 vial	250 µL x ***2*** vials
FAHM-ALZ-1-Item8	Flow Cytometer Setup Bead Cocktail**	200 µL x 1 vial	200 µL x 1 vial
FAX-FB-Item9	Flat-bottom 96-well Microplate	1	1
FAX-VB-Item10	V-Bottom 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. It can also be used to test for bead loss during washing. A detailed procedure for bead loss prevention is outlined in Section VI.F.*
2. *RayBio® Human Serum/Plasma Diluent (Item 4) is a solution that contains similar concentrations of proteins and other components shared by human serum and plasma to mimic the matrix. The diluent included in this kit is a 1:1 PBS-diluted version of the original product (Catalog #137-00022). Larger lots of the original product can be purchased here: https://www.raybiotech.com/human-serum-plasma-diluent-137-00022*
3. *RayBiotech recommends using **a Flat-Bottom 96-well Microplate (Item 9)** in conjunction with an **automated plate washing system** which includes magnetic separation.*

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), Alexa Fluor 700 (AF700), and Alexa Fluor 647 (AF647)/APC channels OR Luminex instrument
- Aluminum foil or 96-well Plate Aluminum Sealers
- Deionized (DI) water
- PBS
- 1.5 mL polypropylene microcentrifuge tubes or similar
- Microcentrifuge
- **Recommended**: Automated plate washing system with magnetic separation
- Optional: 96-well round bottom plate (to prepare samples)
- Optional: High Throughput Sampler (HTS) for 96-well plate reading
- Optional: Vacuum manifold compatible with a 96-well microplate if using a Filter 96-Well Microplate

VI. Assay Protocol

A. Preparation of Samples

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

- **Cell Culture Supernatant / Conditioned Media**
  - **No extra dilution needed, total dilution will be equal to 2-fold 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/
  - 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.
  - For serum-free media, 1X Assay Diluent (Item 2) should be used to dilute samples.

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.*

Note: *If you are using an automatic plate washing system that is not the same as defined in this manual, it is recommended to perform a bead loss prevention check as outlined in Section VI.F.*

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® Magnetic 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 deionized (DI) H_2O to prepare 1X Wash buffer (See Example note below).
4. Dilute the 10X PE Conjugate (Item 7) 10-fold in 1X Assay Diluent (Item 2) to prepare a 1X PE Conjugate 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.

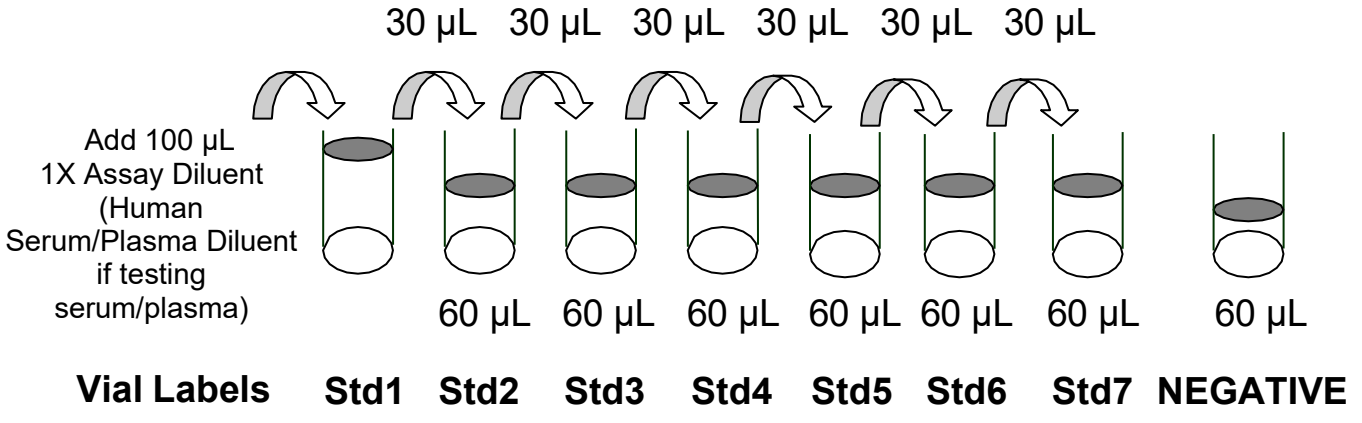
C. Preparation of Protein Standards

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 100 µL 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 or plasma**, add 100 µL RayBio® Human Serum/Plasma 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 or plasma, RayBio® Human Serum/Plasma Diluent (Item 4) should be used to reconstitute and serially dilute the standard mixture.*
3. Obtain six clean 1.5 mL microcentrifuge tubes (or use a 96-well round-bottomplate). Label the tubes/wells as Std2 to Std7. Add 60 µL 1X Assay Diluent (Item 2), or RayBio® Human Serum/Plasma Diluent (Item 4) 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 also image below)

Prepare serial dilutions of protein standards



5. Add 60 µL 1X Assay Diluent (Item 2) or RayBio® Human Serum/Plasma 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 Flat-Bottom 96-well plate (Item 10), V-bottom plate or 1.2 µm filter-plate and mark positions for the standards and samples. Duplicate tests are recommended for all standards and samples.

   Note: *RayBiotech recommends using a Flat-Bottom 96-well plate (Item 10) in conjunction with a magnetic plate washing system. Data in this manual were obtained using a* ***LifeSep™ magnetic separator unit*** *and* ***BioTek 405 TS Washer****. Please note that if alternative systems are used, unforeseen bead loss may occur. If desired, investigators may follow the procedure outlined in Section VI.F to check for bead loss prior to beginning the assay.*

   Note: *A Filter 96-well can be used for the washing steps if desired. The filter plate requires a vacuum manifold compatible with a standard 96-well microplate. Alternatively, a V-Bottom 96-well microplate can be used along with a centrifuge.*

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/plasma 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 using 1X Wash Buffer (*diluted* Item 3; See Section B).

   - **Automated Washing System:** Place the **Flat-Bottom 96-well**

Microplate (Item 9) on the magnetic separator unit for 5 minutes to allow the magnetic beads to migrate to the bottom. Place the Flat-Bottom 96-well Microplate and magnetic separator unit on the platform of an automated plate washer and perform one wash cycle. One wash cycle should include two rounds of dispensing 200 µL of 1X Wash Buffer and aspiration. It is recommended to adjust the settings such that the first aspiration leaves approximately 2 mm of Wash Buffer, and the final aspiration removes all liquid. It is pivotal that all liquid is removed at the end of washing. Remove the plate from the magnetic separator unit once washing is complete. Exact washing procedures may vary depending on the automated system used.

- **V-bottom 96-well Microplate:** Add 200 µL 1X Wash Buffer to each well. Spin the V-bottom microplate down at 1000 g for 5 minutes at room temperature and remove the supernatant using a multichannel pipet. Repeat once more.
- **Filter 96-well Microplate:** Add 200 µL 1X Wash Buffer to each well. 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 once more.

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 as outlined in Step 4.
7. Add 50 µL of 1X PE Conjugate (*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 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 flat-bottom plate, directly read samples from the plate. If using a filter-plate, transfer samples to a V-Bottom plate.
    - 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 Alzheimer's Disease Magnetic Array 1 requires a flow cytometer that is equipped with blue (or green) and red lasers capable of detecting fluorescence in the PE, AF700, and AF647/APC channels or a Luminex instrument. The instructions below describe the set up for a flow cytometer. For Luminex instrument set up, please refer to the instrument manual.*

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, AF700, and AF647/APC compensation may be necessary to decrease the spill-over of AF647/APC signal into PE channel.*

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.

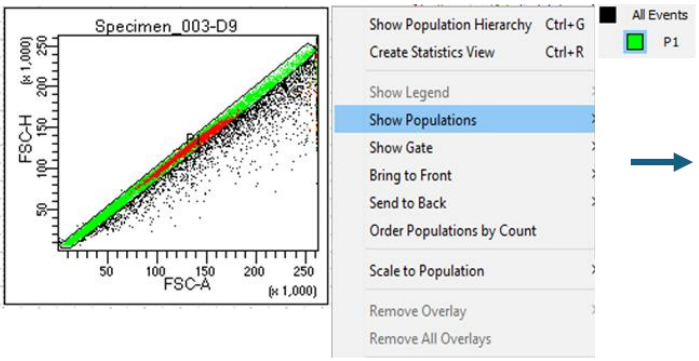
   Note: *The Quality Controls beads are fluorescent particles that are used for flow cytometer setup, e.g., CS&T beads for a BD flow cytometer.*

2. Start a new experiment with PE, AF700, and AF647/APC channels.
3. Resuspend 100 µL of 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 P1, adjust voltage for FSC (forward scatter, linear mode) and SSC (side scatter, linear mode) so that the bead population is clearly defined. Then create a gate around the beads (Figure 3B).
6. Create a new dot plot from the beads parent gate M. Create gates for all bead populations for the assay based on AF700 (log mode) and AF647/APC (log mode). Adjust PMT voltage of AF700 and AF647/APC channels so that all populations are well separated throughout in a prominent area (Figure 3C).

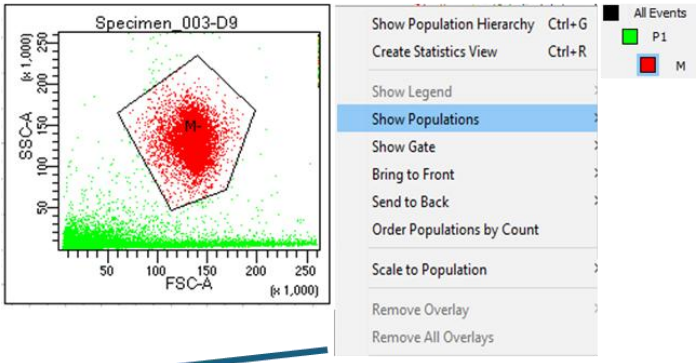
7. Create a statistical view to show the PE MFI for each population (Figure 3D-E).
8. Run a small amount of beads from the NEGATIVE control. Adjust the PE voltage so that the PE median fluorescent intensity (MFI) is around $10^{2\sim3}$ for each bead population.

   Note: *To keep the testing consistent for each assay, we recommend the use of Rainbow Particles, Rainbow Beads, or a similar product to standardize the assay if they are run prior to every analysis. The optimized MFI for both the AF647/APC and PE channels for the Rainbow Particles allow the experiment to be consistent from time to time and machine to machine.*
9. 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.
10. Following setup, run the standards and samples. 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.

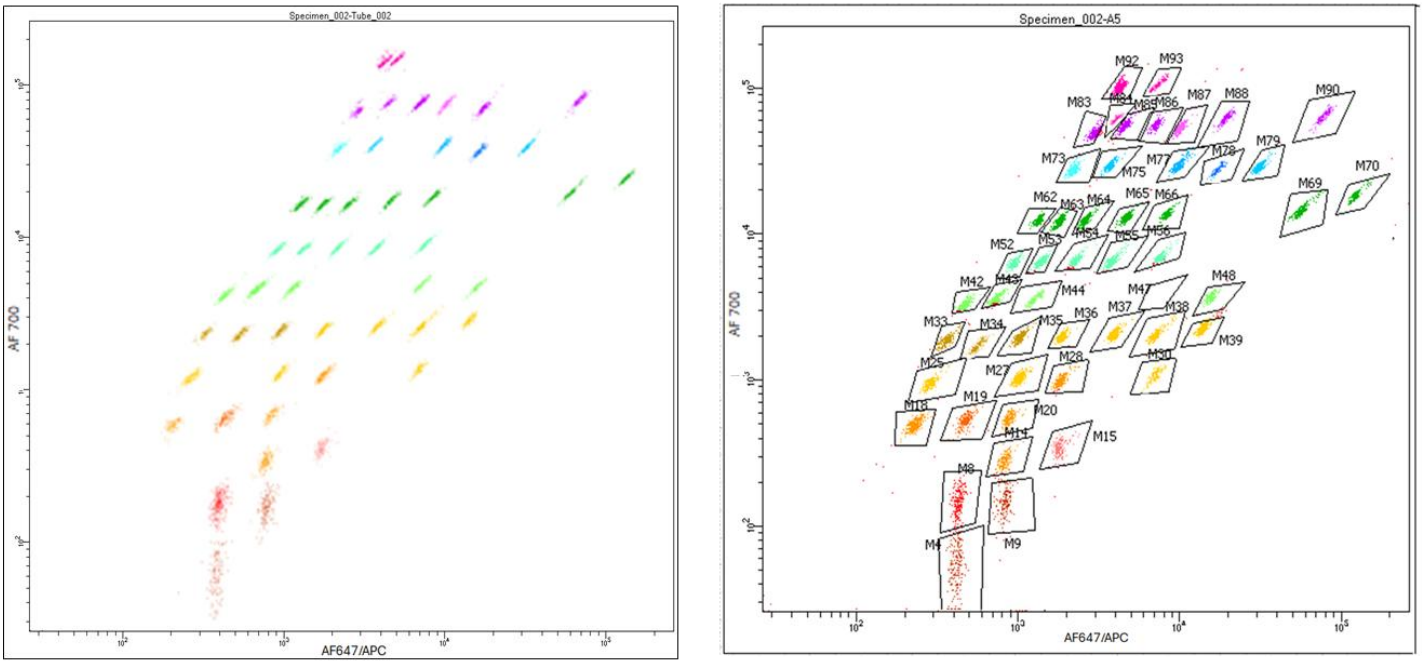
A.



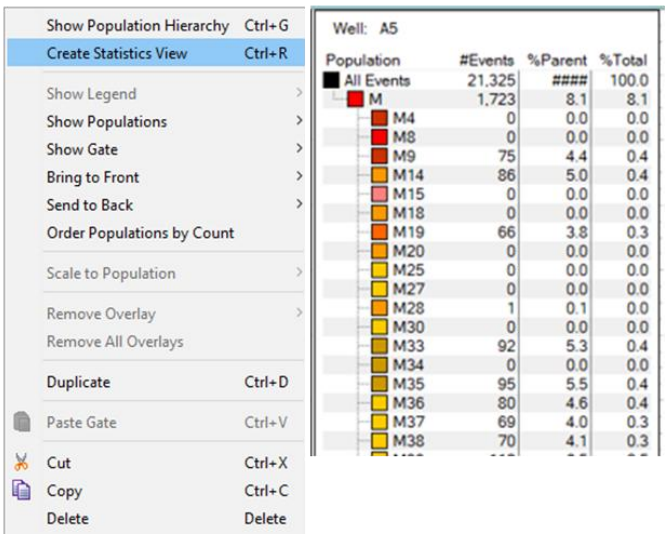
B.



C. Gate



D. Create Statistics View



E. Show PE-A MFI reading

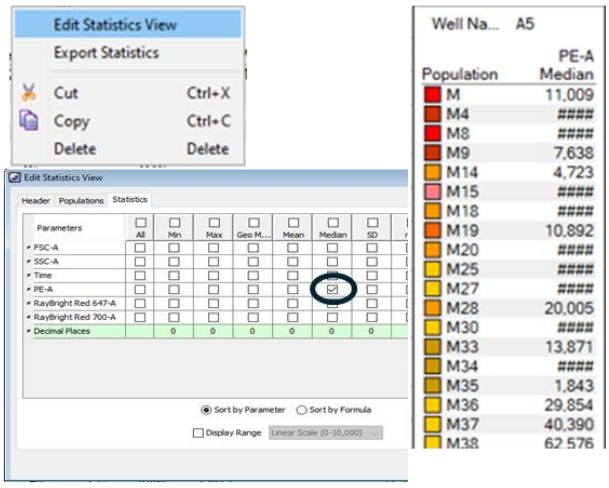


Figure 3. Representative Images of Gated Flow Cytometry Scatter Plots Using RayPlex® Arrays with M-100 series beads. (A) Single beads are used to **(B)** create the parent bead gate M. **(C)** Bead populations are separated with dual fluorescence and gated. **(D, E)** Statistics view is created and edited to show PE-A MFI reading. All PE-A Median values should be selected and exported based on gates of **(C)**.

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

F. Bead Loss Prevention Check

NOTE: *Due to differences in automated plate washing systems, it is recommended to perform a bead loss prevention check prior to starting the assay to prevent loss of beads if an automated washing machine is being used.*

1. Resuspend the contents of **the Flow Cytometer Setup Bead Cocktail (Item 8)** and add 25 µL to one well of a 96-well plate.
2. Add 25 µL of 1X Wash Buffer (*diluted* Item 3) to the same well to mimic the assay protocol.
3. Perform one wash cycle using the automated plate washing system.
    - One wash cycle should always consist of two rounds of 200 µL 1X Wash Buffer being added then removed.
4. Repeat steps 2 and 3 two more times for a total of 3 wash cycles.
5. Resuspend the beads in 150 µL of 1X Wash Buffer (*diluted* Item 3). In normal conditions, 50 µL should have more than 100 events for each bead ID.
6. Run the sample either by transferring to tubes and running in "Tube Mode" or directly from the plate with HTS mode. Optional: Run a sample of unwashed beads to compare beads with and without washing.

    Note: *If the bead count is less than 100 bead events for any bead IDs, it is recommended to reconsider the washing procedure used for the assay. Bead events should ideally be more than 200 for each bead ID. Using a filter-plate for washing will prevent bead loss most effectively*.

G. 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
pTau217	50000.0	16666.7	5555.6	1851.9	617.3	205.8	68.6	0.0
pTau181	50000.0	16666.7	5555.6	1851.9	617.3	205.8	68.6	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.

	pTau217	pTau181
STD 1 (pg/mL)	50000.0	50000.0
Standard 1 MFI	18511.0	15931.7
Standard 2 MFI	14671.7	11651.7
Standard 3 MFI	10764.3	8815.3
Standard 4 MFI	6026.0	4876.0
Standard 5 MFI	2830.7	2159.3
Standard 6 MFI	1408.0	1115.7
Standard 7 MFI	762.3	607.3
Negative MFI	475.3	396.3

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® Human Serum/Plasma Diluent (Item 4).

Target	LOD (pg/mL)
pTau217	11.0
pTau181	18.1

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® Human Serum/Plasma Diluent (Item 4) across 9 replicates. The spiked proteins were assayed, and the measured concentrations were compared to the expected concentrations.

Target	Percent Recovery
pTau217	103.0%
pTau181	97.8%

6. Linearity of Dilution

To test the linearity of dilution, samples (n = 12) were serially diluted in RayBio® Human Serum/Plasma 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 below for data.

Target	Linearity of Dilution
pTau217	104.9%
pTau181	95.5%

7. Intra- and Inter-Assay Precision

1) Intra-Assay Precision

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

Target	Sample #	Mean (pg/mL)	Standard Deviation	CV %
pTau217	1	15945.0	1702.9	10.7%
	2	5297.4	939.6	17.7%
pTau181	1	14773.0	1376.6	9.3%
	2	5373.2	379.4	7.1%

2) Inter-Assay Precision

Spiked standard protein in RayBio® Human Serum/Plasma 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). The inter-assay precision (in CV) of the samples is provided below.

Target	Sample #	Mean (pg/mL)	Standard Deviation	CV %
pTau217	1	17177.3	1501.0	8.7%
	2	5801.2	213.4	3.7%
pTau181	1	16305.1	1620.2	9.9%
	2	6026.4	400.5	6.6%

Note: All data in Section VII were obtained using a flat-bottom 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/plasma samples, prepare the standards in the provided "RayBio® Human Serum/Plasma 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).

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.
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).

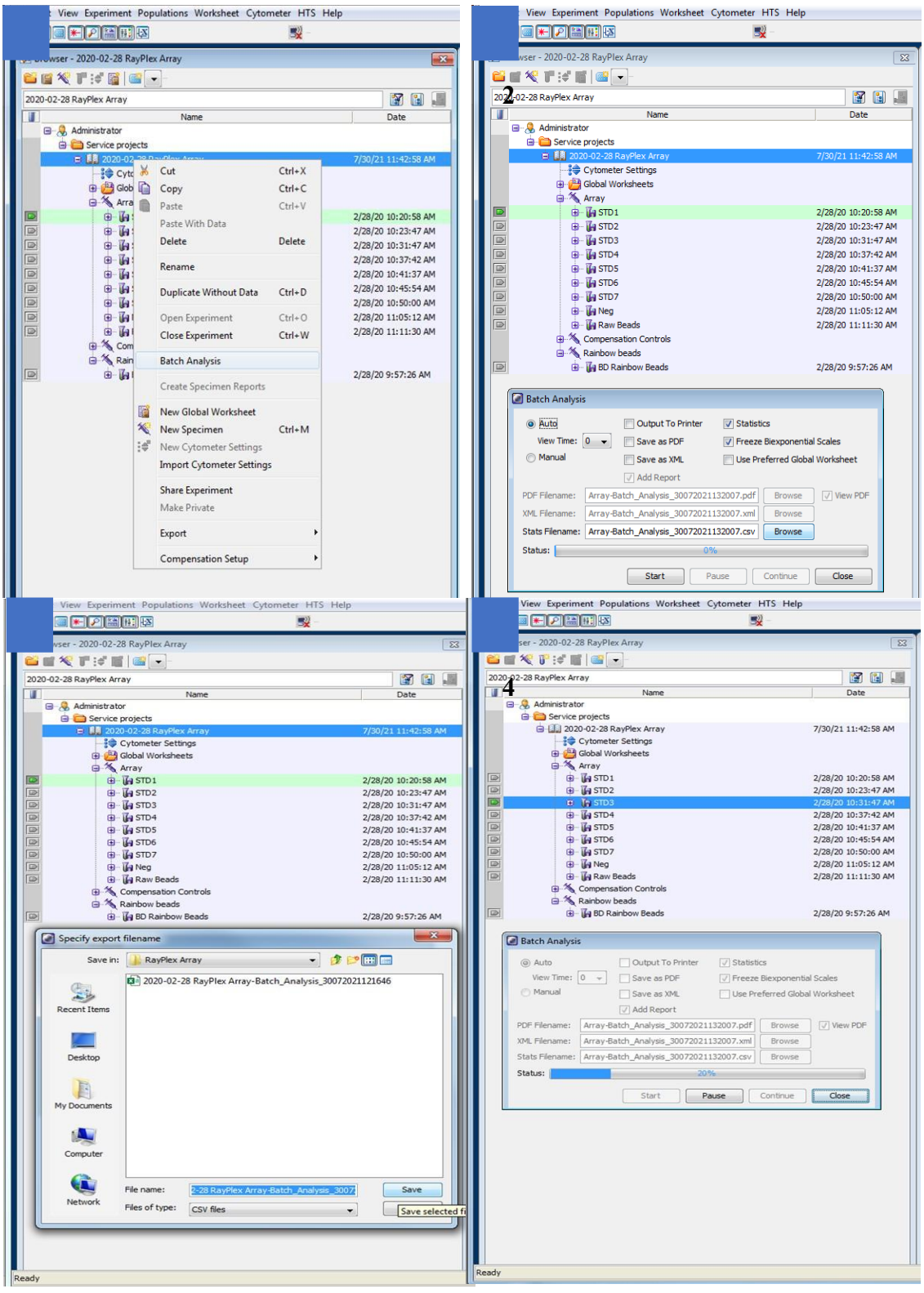


Figure 4. Screen shot showing how to begin the batch analysis through manual tube mode.

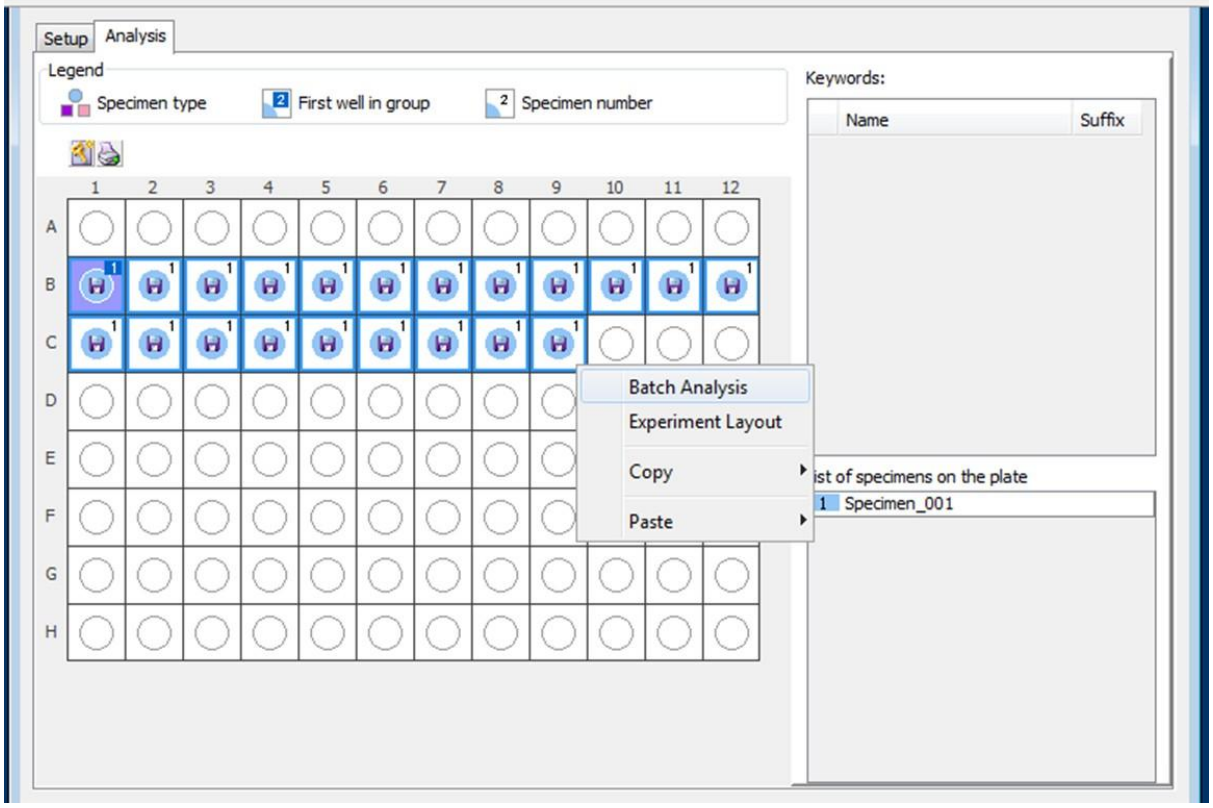
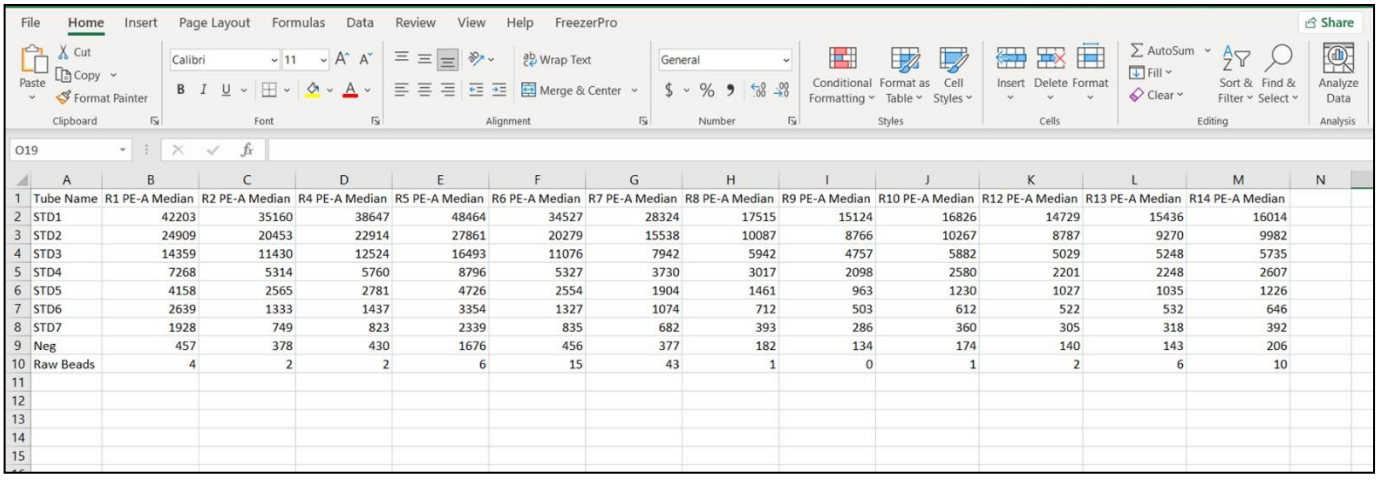


Figure 5. Screen shot showing how to begin HTS batch analysis.



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 6. 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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