AssayGenie

Technical Manual

GeniePlex Multiplex Immunoassays

Research Use Only

GENERAL INFORMATION

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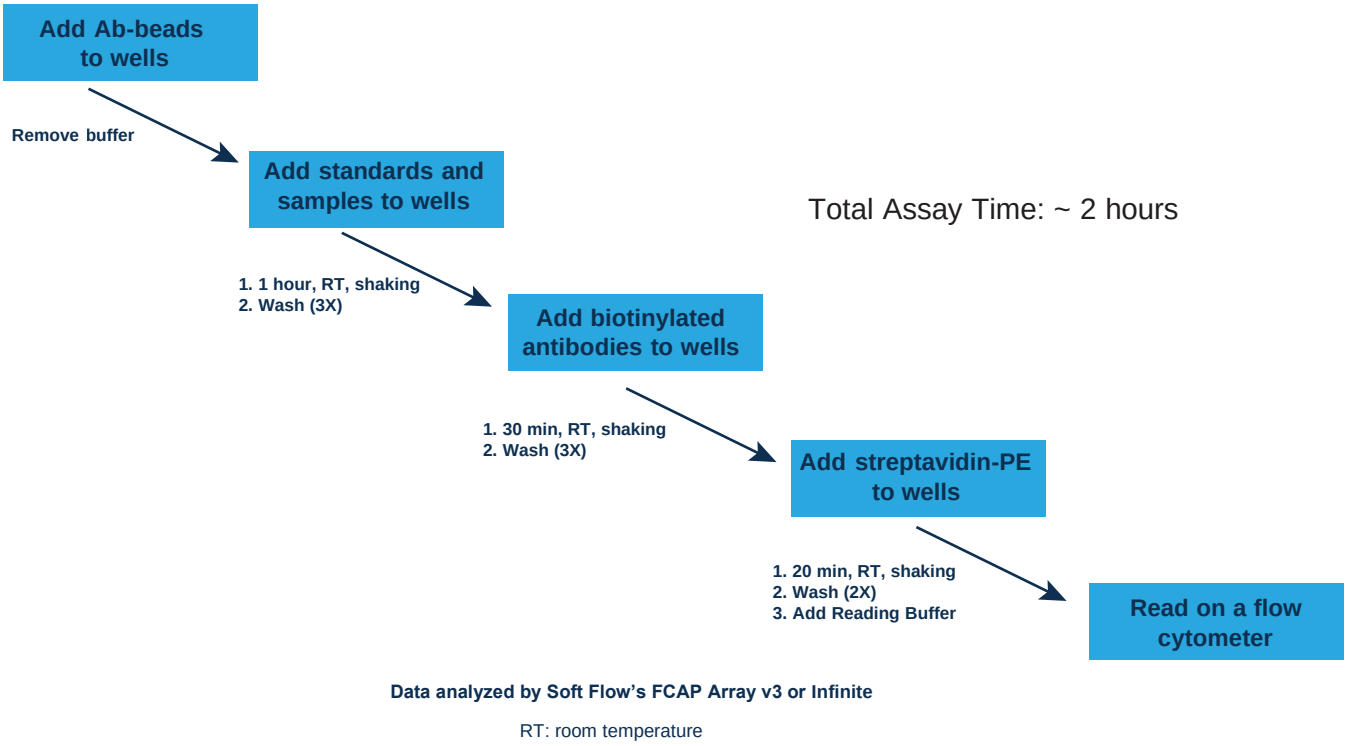
1. DESCRIPTION AND PRINCIPLE

The Assay Genie, GeniePlex multiplex assay technology utilizes multiple bead populations differentiated by size and different levels of fluorescence intensity. With multiple sizes of beads and multiple levels of fluorescence intensity in each bead size, the GeniePlex technology can measure up to 24 analytes simultaneously in a single reaction. The bead populations in the reaction are determined by a flow cytometer equipped with either a single 488nm laser or dual 488nm and 633/640nm lasers. The maximum emission of the bead classification dye is at 700 nm.

Bead-based immunoassays are similar to the principle of a sandwich Assay, having each bead population conjugated with a specific capture antibody trapping the protein of interest, such as a cytokine, in the sample. The amount of the analyte captured is detected via a biotinylated antibody against a secondary epitope of the protein, followed by a streptavidin-R-phycoerythrin treatment. The fluorescent intensity of R-phycoerythrin on the beads is quantified on a flow cytometer. Concentrations of a protein of interest in the samples can be obtained by comparing the fluorescent signals to those of a standard curve generated from a serial dilution of a known concentration of the analyte.

The assay procedure consists of a 60-minute antigen and capture antibody conjugated bead incubation step, a 30-minute biotinylated detection incubation step and a 20-minute streptavidin-PE incubation step.

Assay Protocol Overview:



2. AVAILABLE GENIEPLEX IMMUNOASSAY KIT FORMATS

2.1 Single-Plex Kits

GeniePlex immunoassays are grouped as 12-24 analytes per Group. Each analyte in a Group has a unique bead region and can be multiplexed together in any combination. Analytes in different Groups may be multiplexed together but may have some cross-reactivity because cross- reactivity of all the assays across different groups have not been validated. We offer custom multiplex assay panel design and development. Please contact us for details including pricing and lead time.

NOTE: Bead set assignment may vary in a panel. Refer to the packaging/product inserts and kit labels for specific details.

Each Single-Plex Kit consists of 1 or more user selectable **Analyte Kits**, a **Basic Kit** and a sample matrix specific **Diluent Kit**. Each Analyte Kit contains analyte specific Ab conjugated beads, detection antibodies (dAbs) and corresponding antigen standard(s). The Basic Kit has species-specific detection antibody diluent, reading buffer, wash buffer, Streptavidin-PE (SAPE), 96-well filter plate and plate seals. The Diluent Kit contains sample-type specific Standard Diluent and sample Assay Buffer. Some of the single-plex kits are available in both 96- and 32-test sizes.

2.2 Premixed Multiplex Kits or Kits Packaged as the Premixed Format

Each Premixed Multiplex Kit (or kits packaged as the premixed format) has a pre-defined multiplex panel with premixed antibody-conjugated beads, antigens and detection antibodies. Except the Reading Buffer (10x) and Wash Buffer (10x), all the reagents are supplied as ready-to-use. Some of the premixed multiplex kits are available in both 96- and 32-test sizes.

NOTE: Bead set assignment may vary in a panel. Refer to packaging/product inserts and kit labels for details.

2.3 Custom panel and assay development and custom bulk packaging

We offer custom premixed multiplex panels as well as custom assay development for analytes not currently available. We will work with you to design the panels and assays. Please contact us for pricing, lead time and details of those offerings.

We also offer custom bulk packaging, usually in 5-plate size. Please contact us for details.

3. SUPPLIED REAGENTS & MATERIALS

3.1 Single-Plex Kits

Each Single-Plex Kit consists of 1 or more user selectable Analyte Kits, a Basic Kit and a sample matrix specific Diluent Kit (refer to section 3.3 for available Diluent kits) for 96 tests. Some of the Single-Plex kits are available as 32 tests.

3.1.1 Analyte Kit

Each 96-test Analyte Kit consists of the following components (If multiple analyte kits are ordered, all the components are shipped in a single Analyte Kit box):

Component	Volume
45x Analyte-Specific Ab conjugated beads, e.g. 45x S4P7-human IL-2 (S4 = Size 4µm beads, P7 = Peak 7 of the 4µm beads)	0.1 mL
25x Analyte-Specific biotinylated detection Ab, e.g. 25x Biotin-human IL-2 dAb	0.1 mL
Lyophilized Antigen Standard*	1 vial*

* Each vial may contain one or multiple antigens, refer to lot-specific Standard Info Sheet for details.

Each 32-test Analyte Kit if available consists of the following components (If multiple analyte kits are ordered, all the components are shipped in a single Analyte Kit box):

Component	Volume
45x Analyte-Specific Ab conjugated beads, e.g. 45x S4P7-human IL-2 (S4 = Size 4µm beads, P7 = Peak 7 of the 4µm beads)	35 µL
25x Analyte-Specific biotinylated detection Ab, e.g. 25x Biotin-human IL-2 dAb	35 µL
Lyophilized Antigen Standard*	1 vial*

* Each vial may contain one or multiple antigens, refer to lot-specific Standard Info Sheet for details.

3.1.2 Basic Kit

There are 2 species-specific, non-rodent (NR) and mouse/rat, Basic Kits.

3.1.2.1 NR (Non-Rodent) Basic Kits

These Basic Kits are for human, non-human primate and other non-rodent species assays.

Component	Vol. (32 Tests)	Vol. (96 Tests)
3x NR Detection Ab Diluent	0.3 mL	0.85 mL
1x Streptavidin-PE (SAPE)	1 mL	2.5 mL
10x Reading Buffer	1.5 mL	4 mL
10x Wash Buffer	5 mL	15 mL
PCR 8-tube Strip	2 each	2 each
Filter Plate and Lid	1 each	1 each
Plate Seals	3 sheets	4 sheets
User Manual	1 each	1 each
Packaging insert	1 each	1 each

3.1.2.2 Mouse/Rat Basic Kits

These Basic Kits are for mouse and rat assays.

Component	Vol. (32 Tests)	Vol. (96 Tests)
3x Mouse/Rat Detection Ab Diluent	0.3 mL	0.85 mL
1x Streptavidin-PE (SAPE)	1 mL	2.5 mL
10x Reading Buffer	1.5 mL	4 mL
10x Wash Buffer	5 mL	15 mL
PCR 8-tube Strip	2 each	2 each
Filter Plate and Lid	1 each	1 each

Plate Seals	3 sheets	4 sheets
User Manual	1 each	1 each
Packaging insert	1 each	1 each

3.2 Premixed Multiplex Kits or Kits Packaged as a Premixed Format

Each Premixed Multiplex Kit consists of an Analyte Kit, a Basic Kit for premixed panels and a sample type-specific Diluent Kit (order separately. Refer to section 3.3 for available Diluent kits) for32- or 96-tests.

3.2.1 Analyte Kit

Component	Vol. (32 Tests)	Vol. (96 Tests)
1x Premixed Ab-conjugated beads	1.5 mL	4.7 mL
2x Premixed Biotin-dAbs	0.45 mL	1.25 mL
2x NR or Mouse/Rat dAb Diluent (depending on the assay panel)	0.45 mL	1.25 mL
Lyophilized Antigen Standards* with Standard Info Sheet(s)	1 or multiple vials	1 or multiple vials

* Antigen standards are supplied as premixed but can be in a single vial or multiple vials depending on the panels. Refer to Standard Info Sheet for details.

3.2.2 Basic Kit for Premixed Panels

Component	Vol. (32 Tests)	Vol. (96 Tests)
1x Streptavidin-PE (SAPE)	1 mL	2.5 mL
10x Reading Buffer	1.5 mL	4 mL
10x Wash Buffer	5 mL	15 mL
PCR 8-tube Strip	2 each	2 each
Filter Plate and Lid	1 each	1 each
Plate Seals	3 sheets	4 sheets
User Manual	1 each	1 each
Packaging Insert	1 each	1 each

3.3 Diluent Kits

Diluent Kits are sample type-specific. Please indicate the sample type of your assay panel when placing an order.

3.3.1 CCS (Cell culture supernatant) Diluent kit

This kit is for cell culture supernatant samples. It is universal for all species.

Component	Vol. (96 Tests)
CCS Standard Diluent	2.5 mL
CCS Assay Buffer	5 mL

3.3.2 NR (Non-Rodent) SPB (Serum/Plasma/Bodily Fluid) Diluent kit

This kit is for serum, plasma and bodily fluid (SPB) samples from human, non-human primate and other non-rodent (NR) species.

Component	Vol. (96 Tests)
NR SPB Standard Diluent	2.5 mL
NR SPB Assay Buffer	5 mL

3.3.3 Mouse/Rat SPB (Serum/Plasma/Bodily Fluid) Diluent kit

This kit is for mouse and rat serum, plasma and bodily fluid samples.

Component	Vol. (96 Tests)
Mouse/Rat SPB Standard Diluent	2.5 mL
Mouse/Rat SPB Assay Buffer	5 mL

3.3.4 TL (Tissue/cell lysate) Diluent kit

This kit is for tissue and cell lysate samples. It is universal for all species.

Component	Vol. (96 Tests)
TL Standard Diluent	2.5 mL
TL Assay Buffer	5 mL

4. REQUIRED EQUIPMENT NOT SUPPLIED

- Barnstead/Lab-Line Titer Plate Shaker (Thermo Scientific, Waltham, MA) or equivalent. The shaker should have a 3mm orbit with ability to maintain 600-800 rpm.
- GeniePlex Filter Plate Washer (ACAM00001, https://www.assaygenie.com/genieplex-filter-plate-washer/)
- Vacuum source for the filter plate washer. An economy vacuum pump (e.g. Barnant Model 400-1901; Wikita Model ZK-26 - Oil free diaphragm type vacuum pump pressure 0~0.08mpa, 1000ml; or equivalent) is recommended.
- Flow cytometer capable of detecting forward scatter, side scatter, PE and APC (or PE-Cy5) with Area and Height measurement parameters.

5. PREPARING 1X READING BUFFER AND 1X WASH BUFFER

5.1 1x Reading Buffer

Bring the 10x Reading Buffer to room temperature and vortex for 15 seconds. Mix 4 mL or 1.5 mL of the 10x Reading Buffer with 36 mL or 13.5 mL ddH_20, respectively. The 1x Reading Buffer can be stored at 2-8°C for up to 3 months.

5.2 1x Wash Buffer

Bring the 10x Wash Buffer to room temperature and vortex for 15 seconds. Mix 15 mL or 5 mL of the 10x Wash Buffer with 135 mL or 45 mL ddH_20, respectively. The 1x Wash Buffer can be stored at room temperature for up to 3 months.

6. PREPARING ANTIBODY-COUPLED BEAD WORKING SUSPENSION

6.1 Single-Plex Kits

The capture bead stock provided with each kit is a 45x concentrated stock (1 µL per test). Dilution of the stock beads to a working suspension with 1x Reading Buffer is required.

6.1.1. Determine the number of analytes in the panel (e.g. a 7-plex panel)

6.1.2. Determine the number of wells in the experiment. We recommend adding at least 2 additional wells to account for pipetting recovery. For example, if a total of 48 wells are needed in the experiment, prepare enough for 50 wells.

6.1.3. Determine the total volume of working bead suspension needed for the experiment. Each tube/well needs 45 µL of the working bead suspension. The total volumeis calculated by multiplying the number of wells (calculated in Step 6.1.2.) by 45 µL. For example, 50 wells × 45 µL = 2,250 µL total working bead suspension.

6.1.4. Determine the volume needed for each 45x Analyte-Specific Ab Conjugated Beads (i.e. 1.0 µL needed for each well). Therefore, for example, a total of 50 wells will need 50 µL of each 45x capture bead stock.

6.1.5. Determine the volume of 1x Reading Buffer needed to prepare the working bead suspension. Calculate the 1x Reading Buffer volume by subtracting the volume for each capture bead stock:
____ µL from Step 6.1.3 –___ µL from Step 6.1.4 x ___ number of Plex (Step 6.1.1) = _µL of 1x Reading Buffer to be added.
For example, a 7-plex panel for 50 wells: 2,250 - 50 x 7 = 1900 µL of 1x Reading Buffer

6.1.6. Add the appropriate volume (determined in Step 6.1.5) of 1x Reading Buffer to a test tube labeled with “Working Bead Suspension”.

6.1.7. Centrifuge each capture bead vial at 2,000x g for 10 sec.

6.1.8. Vortex each capture bead vial for 15 second.

6.1.9. Add the appropriate volume (determined in Step 6.1.4.) of each capture bead stock into the “Working Bead Suspension” tube.

6.1.10. Vortex gently to mix. If not used immediately, store the working bead suspension at 2-8°C with light protection. It can be stored at 2-8°C for up to 1 week.

6.2 Premixed Multiplex Kits or Kits Packaged in a Premixed Format

IMPORTANT: The antibody-coupled beads are provided in a ready-to-use 1x suspension. No preparation is needed.

7. PREPARING BIOTINYLATED DETECTION (dAB) ANTIBODY WORKING SOLUTION

7.1 Single-Plex Kits

The Biotinylated Detection (dAb) Antibody provided with each kit is a 25x concentrated stock solution (1 µL per test). Dilution of the biotinylated dAb stock solution to a working solution with NR or Mouse/Rat dAb Diluent is required.

7.1.1. Determine the number of analytes in the panel (e.g. a 7-plex panel)

7.1.2. Determine the number of wells in the experiment. We recommend adding an additional 2 wells to account for pipetting recovery. For example, if a total of 48 wells are needed in the experiment, prepare enough for 50 wells.

7.1.3. Determine the total volume of working dAb solution needed for the experiment. Each tube/well needs 25 µL of the working detection antibody solution. The total volume is calculated by multiplying the number of wells (calculated in Step 7.1.2.) by 25 µL. For example, 50 wells × 25 µL = 1,250 µL total working detection antibody solution.

7.1.4. Determine the volume needed for each 25x Biotinylated Detection Antibody stock (i.e. 1.0 µL needed for each well). Therefore, for example, a total of 50 wells will need 50 µL of each 25x detection antibody stock.

7.1.5. Determine the volume of 3x NR or Mouse/Rat dAb Diluent needed to prepare the working dAb solution.
____ µL from Step 7.1.3 ÷ 3 =___µL of 3x NR or Mouse/Rat dAb Diluent
For example, a total of for 50 wells: 1,250 ÷ 3 = 417 µL of 3x NR or Mouse/Rat dAb Diluent

7.1.6. Calculate the ddH_2O volume by subtracting the volume for each capture bead stock and 3x NR or Mouse/Rat dAb Diluent:
____ µL from Step 7.1.3 –___ µL from Step 7.1.4 x ___ number of Plex (Step 7.1.1) µL from Step 7.1.5 =___µL of ddH_2O
For example, a 7-plex panel for 50 wells: 1,250 - 50 x 7 - 417 = 483 µL of ddH_2O

7.1.7. Add the appropriate volume (determined in Step 7.1.6) of ddH_2O to a test tube labeled with “Working dAb Solution”

7.1.8. Add the appropriate volume (determined in Step 7.1.5) of 3x NR or Mouse/Rat dAb Diluent to the “Working dAb Solution” tube.

7.1.9. Add the appropriate volume (determined in Step 7.1.4.) of each dAb stock into the “Working dAb Suspension” tube.

7.1.10. Mix by gentle vortexing. The working dAb solution can be stored at 2-8°C for up to 24 hrs.

7.2 Premixed Multiplex Kits or Kits Packaged in a Premixed Format

IMPORTANT: Prepare a working solution by transferring the entire content of the 2x NR or Mouse Rat dAb diluent to the 2x Biotin-dAb vial. Mix by gentle vortexing. The working solution can be stored at 2-8°C for up to 24 hrs.

NOTE: If running a partial plate, calculate the amount of the working solution needed by multiplying the number of wells by 25 µL. We recommend adding an additional 2 wells to account for pipetting recovery. For example, if a total of 48 wells are needed in the experiment, prepare enough for 50 wells (50 wells × 25 µL = 1,250 µL total working detection antibody solution). Prepare the working detection antibody solution by combining 625 µL of 2x NR or Mouse/Rat dAb diluent and 625 µL of the 2x Premixed Biotin-dAbs.

8. PREPARING ANTIGEN STANDARDS

Note: Reconstitute and prepare serial dilutions of the antigen standards no more than 2 hours before carrying out the assay(s) in Section 9. Discard after use. Freezing, thawing and reuse of there-constituted and diluted standards are **NOT** recommended.

Note: If running a partial plate, extra vials of antigen standards should be ordered.

8.1 Reconstitution of the lyophilized standards

8.1.1 If there is only ONE standard vial in the kit

8.1.1.1. Centrifuge the antigen standard vial at 2,000x g for 10 sec.

8.1.1.2. Add 250 µL of CCS (cell culture supernatant), SPB (serum/plasma/bodily fluid) or TL (Tissue/cell lysate) standard diluent into the vial.

NOTE: Reconstituted volume may be different for custom assay panels. Refer to the "**Standard Info Sheet**" enclosed in the kit for details.

8.1.1.3. Vortex gently for 15 sec.

8.1.1.4. Incubate on ice for 5-10 min.

8.1.1.5. Vortex gently for 15 secs before the Serial Dilution Preparation (Step 8.2)

8.1.2 If there are MULTIPLE standard vials in the kit

8.1.2.1. Centrifuge the antigen standard vials at 2,000x g for 10 sec.

8.1.2.2. Add 250 µL of CCS (cell culture supernatant), SPB (serum/plasma/bodily fluid or TL (Tissue/cell lysate) standard diluent into the first vial.

NOTE: Reconstituted volume may be different for custom assay panels. Refer to the **"Standard Info Sheet**" enclosed in the kit for details.

8.1.2.3. Vortex gently for 15 secs

8.1.2.4. Incubate on ice for 5 min

8.1.2.5. Vortex gently for 15 secs

8.1.2.6. Transfer the entire content to the next vial

8.1.2.7. Vortex gently for 15 secs

8.1.2.8. Incubate on ice for 5 min

8.1.2.9. Vortex gently for 15 secs

8.1.2.10. If more than 2 standard vials in the kit, repeat Steps 8.1.2.6 to 8.1.2.9 for the rest of the vials.

8.2 Serial dilution preparation

Prepare 3x serial dilutions (160 µL in total, enough for triplicate wells) according to Table 1. Mix each addition by pipetting up and down 6–8 times. Change pipette tips after each addition to avoid contamination from one concentration to the other. Keep the standards on ice until use.

Table 1: Preparation of antigen Standard Curve

Standard	Amount from Previous Standard (µL)	Standard Diluent (µL)
Standard 1 (Undiluted)		Prepared in Section 8.1
Standard 2 (1/3)	80	160
Standard 3 (1/9)	80	160
Standard 4 (1/27)	80	160
Standard 5 (1/81)	80	160
Standard 6 (1/243)	80	160
Standard 7 (1/729)	80	160
Blank	0	160

NOTE: If very low levels (less than 10 pg/mL) of cytokines are expected in the samples, we recommend adding 1 or even 2 more standard points, e.g. Standard 8 (1/2187) andStandard 9 (1/6561) in the assay for better quantitation at low concentrations. Standard 1 may be omitted when assaying those samples.

NOTE: Serial dilution factor may be different for custom assay panels. Refer to the **"Standard Info Sheet"** enclosed in the kit for details.

9. PERFORMING THE ASSAY

9.1. Prepare the filter plate template. Mark the standard, sample and blank wells. Standards and samples should be run in duplicates or triplicates. If the whole plate will not be used, seal the unused well with a plate seal.

IMPORTANT: Place the filter plate on top of the filter plate lid during the entire assay process to prevent touching the plate bottom on any surface.

9.2. Vortex working bead suspension for 15 seconds.

9.3. Add 45 µL of capture bead working suspension to each well.

NOTE: Save the remaining capture bead working suspension and store at 2-8°C with light protection. It can be used for setting up acquisition parameters on the flow cytometer.

9.4. Remove buffer in the wells by using the "flow-through" Filter Plate Washer connected to a vacuum source that has been adjusted according to the Filter Plate Washer Instructions.

9.5. Gently tap the plate bottom onto several layers of paper towels to remove residual buffer after the "flow-through" removal of the buffer.

9.6. Add 30 µL of CCS, SPB or TL Assay Buffer to each sample well.

NOTE: Cell culture supernatant samples can be run without diluting in Assay Buffer if very low levels (less than 20 pg/mL) of cytokines are expected. If it is the case, skip this step and add 45 µL of cell culture supernatant samples to each sample well in Step 9.7.

9.7. Add 15 µL of samples to each sample well.

9.8. Add 45 µL of standards to each standard well.

9.9. Cover the plate with a plate seal.

9.10. Incubate on the shaker (set at 700 rpm) for 60 min at room temperature. Protect from light by wrapping the filter plate in aluminum foil.

9.11. Remove the plate seal.

9.12. Remove solutions in the wells by using the Filter Plate Washer connected to a vacuum source.

9.13. Wash the wells three times with 100 µL 1x Wash Buffer using the Filter Plate Washer.

9.14. Gently tap the plate bottom onto several layers of paper towels to remove residual buffer on the plate bottom after the last wash.

9.15. Add 25 µL of biotinylated antibody working solution to each well.

9.16. Cover the plate with a plate seal.

9.17. Incubate on the shaker (set at 700 rpm) for 30 min at room temperature. Protect from light by wrapping the filter plate in aluminum foil.

9.18. Remove the plate seal.

9.19. Remove solutions in the wells by using the Filter Plate Washer.

9.20. Wash the wells three times with 100 µL 1x Wash Buffer using the Filter Plate Washer.

9.21. Gently tap the plate bottom onto several layers of paper towels to remove residual buffer on the plate bottom after the last wash.

9.22. Add 25 µL of streptavidin-PE working solution to each well.

9.23. Cover the plate with a plate seal.

9.24. Incubate on the shaker (set at 700 rpm) for 20 min at room temperature. Protect from light by wrapping the filter plate in aluminum foil.

9.25. Remove the plate seal.

9.26. Remove solutions in the wells by using the Filter Plate Washer.

9.27. Wash the wells twice with 100 µL 1x Wash Buffer.

9.28. Gently tap the plate bottom onto several layers of paper towels to remove residual solution.

9.29. Add 150 µL to 300 µL of 1x Reading Buffer to each well depending on the sample loading mechanism of a flow cytometer to re-suspend the beads.

9.30. Cover the plate with a plate seal.

9.31. Place the plate on the microtiter shaker and shake for 30 seconds at 700 rpm.

NOTE: If the flow cytometer has no 96-well plate loader and more than 200 µL of 1x Reading Buffer is needed to re-suspend the beads, do not shake the plate. Re-suspend the beads in each well by pipetting up and down 6–8 times with a P200 pipette then transfer to a test tube for acquisition.

9.32. Remove the plate seal.

9.33. Read on a flow cytometer by following Section 10.

NOTE: If the assayed plate is not read immediately, it can be stored at 2-8°C for up to 16 hr. The plate should be sealed with a plate seal and protected from light by wrapping the filter plate in aluminum foil.

10. SETTING UP FLOW CYTOMETERS

10.1 Fluorescence channels

The maximum emission of the bead classification dye is at 700 nm. It can be detected on “PE-Cy5“ channels of most of the flow cytometers with blue (488 nm) excitation. It can also be detected on **PE-Cy7 channels with blue (488 nm) excitation or APC channel with red (633 or 640nm) excitation** if such a florescence channel is available.

The reporter dye of the GeniePlex assays is PE and can be detected on the PE channel with blue (488 nm) excitation.

10.2 Preparing instrument setup beads

10.2.1. BLANK BEADS: Aliquot half (e.g. 75 µL if resuspension volume in Step 9.29 is 150 µL) of bead suspension from one of Blank wells from Step 9.32 into a sample tube or a well of a 96-well plate depending on the sample loading mechanism of a flow cytometer. Add 100 to 300 µL of 1x Reading Buffer to the tube/well.

NOTE: Remaining capture bead working suspension from Step 9.3 can also be used for this purpose. Aliquot 45 µL of the remaining capture bead working suspension from Step 9.3 into a sample tube or a well of a 96-well plate depending on the sample loading mechanism of a flow cytometer. Add 100 to 500 µL of 1x Reading Buffer to the tube/well.

10.2.2. STANDARD 1 BEADS: Aliquot half (e.g. 75 µL if resuspension volume in Step 9.29 is 150 µL) bead suspension from one of the Standard 1 wells from Step 9.32 into a sample tube or a well of a 96-well plate depending on the sample loading mechanism of a flow cytometer. Add 100 to 300 µL of 1x Reading Buffer to the tube/well.

NOTE: Add 75 µL - 150 µL of 1x Reading Buffer to the Blank and Standard 1 wells. Acquisition for both wells will be slower (less bead concentrations) during the sample acquisition step. **IMPORTANT:** When running a panel the first time, we recommend running one extra well of Standard 1 to provide proper instrument setup prior to running all standards and samples.

10.3 Setting up a display layout/template

10.3.1. Perform instrument start up and verification of fluidic stability and optical alignment by following cytometer manufacturer‘s recommendations including running calibration and/or performance beads, such as Spherotech’s Rainbow Calibration Particles (Rainbow Calibration Particles.pdf).

10.3.2. Open a new protocol.

10.3.3. Create the following plots and histograms:

1) A dot plot with FS (X-axis) and SS (Y-axis) in linear display mode.

2) 2 histograms of “ PE-Cy5“ in Log display mode.

3) 2 dot plots with PE (X-axis) and “PE-Cy5“ (Y-axis) in Log display mode.
4) If PE-Cy7 or APC channel is available, create 2 histograms of “PE-Cy5“ in Log display mode and 2 dot plots with PE (X-axis) and “PE-Cy7“ or “APC“ (Y-axis) in Log display mode.

10.3.4. Set all compensation to zero.

10.3.5. Both Areas and Heights, if available, of the FL parameters should be collected.

10.3.6. Save the protocol.

10.4 Running the setup beads

10.4.1. Run the **BLANK BEADS** prepared in Step 10.2.1.

1) Adjust FS and SS gains so that the bead populations are on scale (Figure 1).
2) Create Gate 1 for the smaller (4-micron size, S4) beads and Gate 2 for the larger (5-micron size, S5) beads (Figure 1).

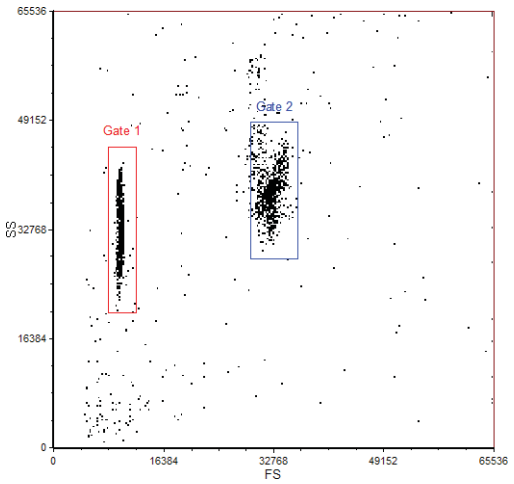


Figure 1

3) Gate 1 to one of the “PE-Cy5“ histograms and one of the PE/“PE-Cy5“ dot plots.
4) Apply Gate 2 to the other “PE-Cy5“ histogram and the other PE/“PE-Cy5“ dot plot
5) Adjust “PE-Cy5“ PMT voltage so that all the bead populations are clearly separated on the histograms and dot plots (Figure 2).
In this example, S4 has 4 bead populations, S4P3, S4P7, S4P9 and S4P11. S4P3 (Size 4-micron, Peak #3) is the dimmest and S4P11 (Size 4-micron, Peak #11) is the brightest.
6) Adjust PE PMT voltage so that the dimmest bead population is positioned within the first decade on the PE-axis of the plot (Figure 2).

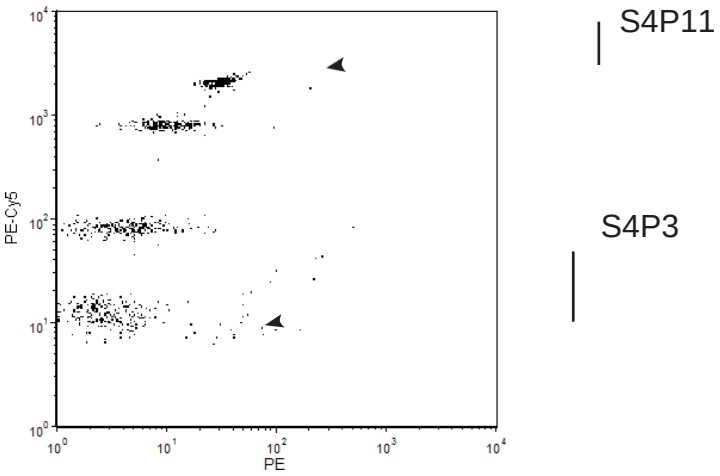


Figure 2

7) Save the protocol.

10.4.2. Run the Standard 1 beads prepared in Step 10.2.2.

1) Verify all the bead populations on the PE-axis are on scale (Figure 3).

2) Adjust PE PMT voltage if needed. If adjustment is needed, make sure re-run the Standard 8 and/or make sure the dimmest bead population is still visible on the PE/“PE-Cy5“ dot plots.

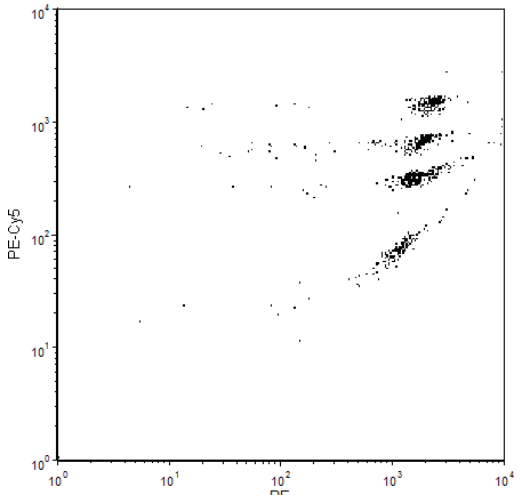


Figure 3
Uncompensated

3) Apply proper “PE-Cy5“ - %PE color compensation so that the bead populations are in a horizontal position (Figure 4: small blue circle is an example of proper color compensation setting).

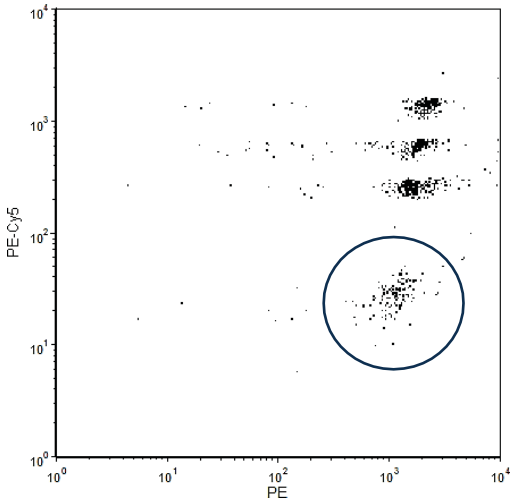


Figure 4
Proper compensation

Over-compensation should be avoided (Figure 5: large blue circle is an example of over-compensation).

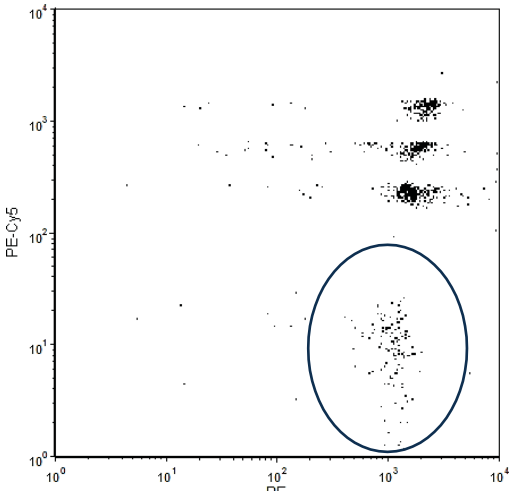


Figure 5
Improper compensation

4) Save the protocol.

Note: If PE-Cy7 or APC fluorescence channel is available, carry out Steps 10.4.1 and 10.4.2 for the PE-Cy7 or APC channel for the proper PMT voltage and color compensation (usually zero) settings.

11. ANALYZING THE SAMPLES

If the flow cytometer is not equipped with a 96-well plate loader, re-suspend the beads in a well by pipetting up and down 6–8 times then transfer to a test tube for acquisition. Acquire 100 events for each bead population of the larger beads (Gate 2). For example, if there are 3 bead populations in Gate 2 (larger beads), acquire 3 x 100 = 300 events per sample. We have found that as few as 50 events for a bead population are sufficient. Save the FCS files for data analysis.

FCAP Array v3.0 or Infinite (Soft Flow, Inc. https://softflow.com/EN/en_fcap.html) are recommended for data analysis.

12. TECHNICAL HINTS

12.3.1. Set up the Filter Plate Washer according to the instruction in the Product Insert. Adjust the vacuum pressure so that 100 µL of 1x Wash Buffer in the wells can be clear in 3-5 second. (DO NOT USE a standard ELISA plate washer for GeniePlex assays unless specifically designed for “flow through” non-magnetic beads.)

12.3.2. When working with samples and standards change the pipette tips after every transfer and avoid creating bubbles when pipetting.

12.3.3. We recommend, whenever possible, using a multi-channel pipette for reagent additions to achieve optimal assay precision.

12.3.4. When applying a plate seal to the filter plate, do not use a rubber roller to seal it. Use a finger to gently press over the plate seal to seal the plate.

12.3.5. Sample handling:

a. **Cell Culture Supernatant:** Remove particles by centrifugation and assay immediately or aliquot and store samples at ≤ -80°C. Avoid repeated freeze-thaw cycles.

b. **Serum:** If using serum separator tubes, allow samples to clot for 30 minutes at room temperature. Centrifuge for 10 minutes at 1,000x g.

Collect the serum fraction and assay promptly or aliquot and store the samples at -80°C. Avoid multiple freeze-thaw cycles. If serum separator tubes are not being used, allow samples to clot overnight at 2-8°C. Centrifuge for 10 minutes at 1,000x g. Remove serum and assay promptly or aliquot and store the samples at -80°C. Avoid multiple freeze-thaw cycles.

c. Plasma: Centrifuge samples at 1,000x g at 4°C for 10 min within 30 min of blood collection. Collect the plasma fraction and assay promptly or aliquot and store the samples at -80°C. Avoid multiple freeze-thaw cycles.

d. Thaw frozen samples on ice and mix well prior to adding to the assay wells.

e. Cytokines and proteins can be degraded after freezing and thawing. Whenever possible, immunoassays should be conducted with freshly prepared samples especially with those containing low analyte concentrations.

f. If there is a high lipid content in serum, plasma or bodily fluid samples, centrifuge at 10,000 x g for 10 min at 2-8°C. Collect the serum, plasma or bodily fluid fraction for the assays.

g. If samples contain high analyte concentrations and need dilution for the assays, use Sample Dilution Buffer for sample dilution. The exact dilution must be determined empirically.

12.3.6. If 30 µL of Assay Buffer and 15 µL of sample are added to a well, dilution factor is 3 when calculating the final concentration.

13. TROUBLESHOOTING

Issue	Possible cause	Recommended actions
Low event count	Beads aggregate	Vortex stock and working bead suspensions well before pipetting.
	Beads settle on the well bottom	Shake plates at 700 rpm for 30 seconds prior to acquisition or re-suspend the beads in a well by pipetting up and down 6–8 times with a P200 pipette prior to transferring to a sample tube for acquisition.
	Vacuum too strong	Adjust the vacuum pressure so that 100 µL of 1x Wash Buffer in the wells can be clear in 3-5 second.
Low assay signal or sensitivity	Standard not reconstituted well	Standard(s) should be incubated on ice for 5min after the addition of standard diluent.
	Incubation time too short	Follow recommended incubation time in each step.
	Excess exposure to light	During incubation, cover the plate with aluminum foil to minimize exposure of the beads to light.
High Background	Well-to-well contamination	Change pipette tips after every transfer. Remove plate seal carefully and avoid contents from one well to mix with another.
Low Precision	Poor pipetting precision	Use calibrated pipettes.
	Contamination from adjacent wells	Avoid well-to-well contamination during pipetting and removal of plate seal.
Clogged filter plate	High lipid content in the serum, plasma or bodily fluid samples	Centrifuge the samples at 10,000 x g for 10 min at 2-8°C. Collect the serum, plasma or bodily fluid fraction.

NOTES

Where to find us:

Email: info@assaygenie.com

Phone: + (44) 2081237624

Address: 2 Duke Street
London
United Kingdom
W1U 3EH

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