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Muse® P13K/MAPK Dual Pathway Activation Kit User's Guide

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For Research Use Only. Not for use in diagnostic procedures.
MCH200108MAN, Rev B
Catalog No. MCH200108 (50 tests)
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Introduction

Muse® PI3K/MAPK Dual Pathway Activation Kit is designed to enable researchers a quick and easy way to cross examine both the PI3K and MAPK signaling pathways simultaneously using the Guava® Muse Cell Analyzer. Recent evidence suggests that there is cross-talk between the PI3K and the MAPK signaling pathways. Antibodies against phosphorylated Akt (pAkt) and phosphorylated ERK (pERK) can be used to examine PI3K/MAPK interactions. Data generated using the Guava Muse Cell Analyzer along with the corresponding Muse software module provides statistical values measuring:

- Percentage of negative cells (e.g., no PI3K or MAPK pathway activation)
- Percentage of ERK1/2 activated cells (MAPK pathway activation)
- Percentage of Akt activated cells (PI3K pathway activation)
- Percentage of dual pathway activation (MAPK and PI3K pathway activation)

Examination of cell signaling pathways and monitoring their activation status have been extremely important for researchers to understand the detailed mechanisms of cellular functions and the cause of various diseases. Many signal transduction pathways have been implicated to lead to multiple outcomes such as apoptosis, cell differentiation, cell growth and cell proliferation, all of which have been extensively studied for the treatment of various cancers and autoimmune diseases.

The study of cell signaling pathways has been made easier with the use of activation status-specific and phospho-specific antibodies. Measurement of protein phosphorylation with phospho-specific antibodies has given insight into kinase signaling cascades.[1] Multi-parameter phospho flow cytometry is a powerful tool for studying multiple pathways in a mixed cell population at the same time.

Much excitement in the field of signal transduction has centered on the discovery of increasing crosstalk among signaling pathways.[2] Recent evidence has suggested that communication between the PI3K and MAPK pathways exist downstream from the cell surface.[2–4] The ability for signaling pathways to cross-talk adds an extra dimension and complexity when evaluating pathways of interest. Since signal transduction pathways are an elaborate highway of events, the ability to monitor these key intracellular "checkpoints" simultaneously provides researchers a very powerful tool for analyzing complicated cell events such as cancer cell proliferation by measuring the activity of multiple cell signaling pathways.

The Muse PI3K/MAPK Dual Pathway Activation Kit is optimized on the Guava Muse Cell Analyzer. Both antibodies provided in the kit are carefully titrated and optimized together to ensure maximal performance when run in multiplex, alleviating the need for any additional optimization. This kit contains optimized fixation, permeabilization, and assay buffers to provide researchers with a complete solution for PI3K and MAPK cell signaling analysis.

Sufficient reagents are provided to perform 50 tests. Detailed assay instructions are included to assist in analysis and to ensure the correct cell concentration is obtained during acquisition of sample data.

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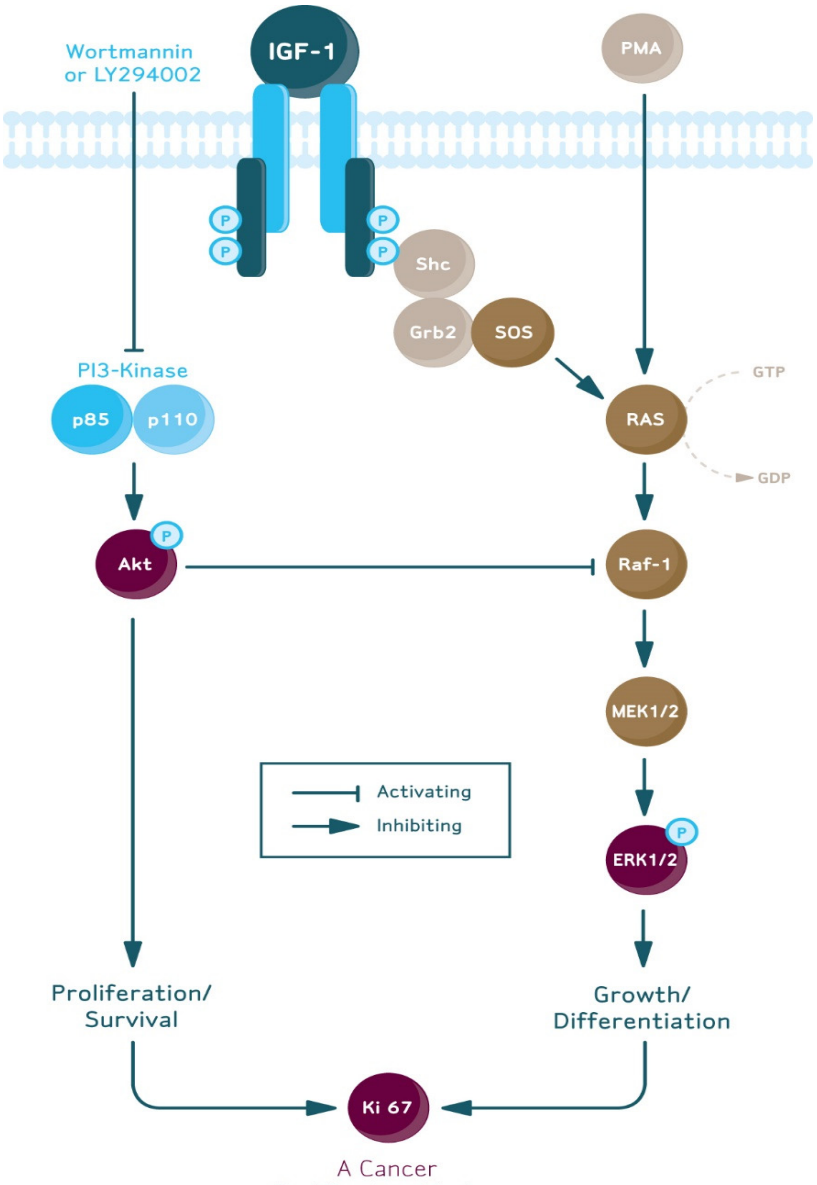
Product Overview

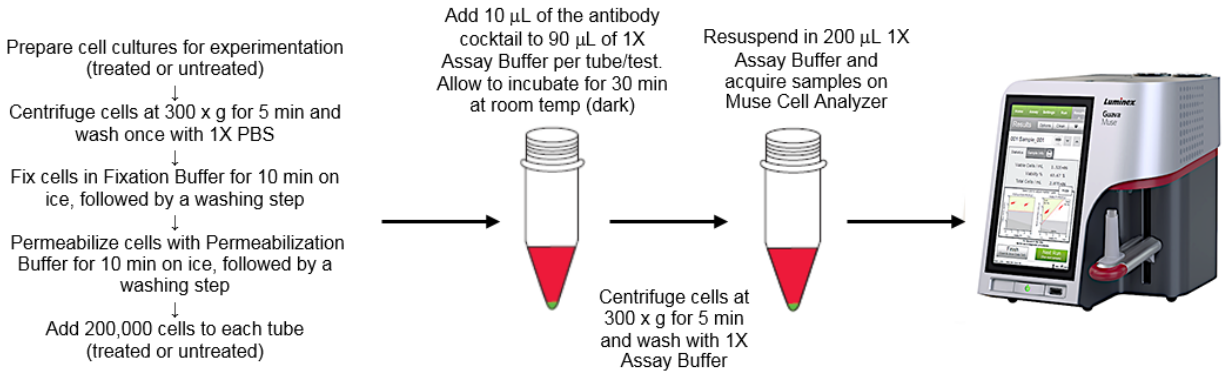
The Muse® PI3K/MAPK Dual Pathway Activation Kit includes two directly conjugated antibodies, a phospho-specific Akt (Ser473)-Alexa Fluor™ 555 and a phospho-specific ERK1/2 (Thr202/Tyr204, Thr185/Tyr187)-PECy5 conjugated antibody to assess the activation of both the PI3K and MAPK signaling pathways simultaneously in testing samples. Together, two-color analysis of Akt and ERK1/2 activation in multiplex provides a reliable detection method for assessing the interactions and possible cross-talk activities for these two key signaling pathways.

Anti-phospho-Akt and Anti-phospho-ERK1/2 antibodies are provided in the kit to measure cell-based PI3K and MAPK activity. It has been suggested that the phosphorylation of Akt can result in the inhibition, or dephosphorylation, of phospho-Raf on Ser 259.[2] By inactivating Raf, this will essentially block the MAPK signaling pathway resulting in an inactivated phospho-ERK. In some situations, a surface receptor (such as IGF-1) will activate both the PI3K and MAPK pathways leading to the phosphorylation of both Akt and ERK. However, since there is cross-talk between the two pathways, it is critical to investigate their interactions in both a spatial and temporal manner.

Figure 1: Cross-talk between the PI3K/MAPK signaling pathways Evidence suggests that the PI3K and Ras pathways can intersect by cross-talk among their downstream effectors. Muse PI3K/MAPK Dual Pathway Activation Kit provides two essential phospho-specific antibodies for the researcher to fully interrogate the relationship between the PI3K and MAPK pathways, phospho-Akt and phospho-ERK1/2, respectively.



Summary of Protocol



Materials Provided

- 20X Anti-phospho-Akt (Ser473), Alexa Fluor™ 555 (Part No. CS208168). One vial containing 250 µL
- 20X Anti-phospho-ERK1/2 (Thr202/Tyr204,Thr185/Tyr187), PECy5 (Part No. CS208167). One vial containing 250 µL
- 5X Assay Buffer (Part No. CS202124). One bottle containing 55 mL
- Fixation Buffer (Part No. CS202122). One bottle containing 13 mL
- 1X Permeabilization Buffer (Part No. CS203284). One bottle containing 14 mL

Materials Required but Not Supplied

Reagents/Consumables

- Test tubes for sample preparation and storage
- Tissue culture reagents, i.e., HBSS, PBS w/o Ca^{2+} or Mg^{2+}, cell dislodging buffers, etc.
- Deionized water (for buffer dilution)
- Cells of interest in suspension (e.g., HeLa, HEK293, etc.)
- Microcentrifuge tubes with screw caps, 1.5 mL (VWR, Catalog No. 16466-030, or equivalent)
- Muse® System Check Kit (Catalog No. MCH100101)
- Guava® ICF Instrument Cleaning Fluid (Catalog No. 4200-0140), optional

Equipment

- Pipettes with corresponding tips capable of accurately measuring 10–1000 µL
- Tabletop centrifuge capable of achieving 300 xg
- Mechanical vortex
- Guava® Muse® Cell Analyzer

Warnings and Precautions

- The instructions provided have been designed to optimize the kit's performance. Deviation from the kit's instructions may result in suboptimal performance and may produce inaccurate data.
- Some assay components included in the kit may be harmful. Please refer to the Safety Data Sheet (SDS) for specific information on hazardous materials (SDSs can be found on the web page or by contacting Luminex Technical Support).
- During storage and shipment, the directly conjugated antibodies may condense within the vial. For maximum recovery of the product, centrifuge original vial prior to removing cap.
- The conjugated antibody is light sensitive and must be stored in the dark at 2–8°C.
- Do not use reagents beyond the expiration date of the kit.

Storage

All reagents must be stored at 2–8ºC.

Please refer to your product label for information regarding the product lot, including the expiration date. Details on the Luminex Reagent Shipping Policy, including any shelf-life guarantees, can be found at https://www.luminexcorp.com.

Before You Begin

It is highly recommended that you run the cell samples shortly after completing the sample preparation. While some cell types have been shown to yield stable results for up to 24 hours after cell fixation/permeabilization/antibody staining if properly stored, the stability of individual cell types may vary.

Time considerations: When dealing with phospho-specific activation detection, fixation of cell samples after cell treatment(s) is critical to capture the phosphorylation/activation event. Some activation state cell signaling responses are transient and may be lost if cell cultures are not fixed immediately following treatment. Cell fixation, permeabilization, and staining will take approximately 50 minutes. Acquiring data on your Guava® Muse® Cell Analyzer takes less than 3 minutes per sample, depending on the cell concentration and desired number of events to acquire.

Always run a System Check prior to performing the assay. For details, refer to the *Guava Muse Cell Analyzer User's Guide*.

Preparation of Reagents

1. **Assay Buffer**

   Assay Buffer is supplied at 5X concentration and should be diluted to 1X with deionized water prior to use. Prepared 1X Assay Buffer is stable up to 1 year. Store at 2–8°C.

2. **Antibody Working Cocktail Solution**

   The kit contains two antibodies which can be used in multiplex. Prior to antibody staining of cells, prepare an antibody working cocktail solution by addition of the following: Add 5 µL of anti-phospho-Akt (Ser473), Alexa Fluor™ 555 and 5 µL of anti-phospho-ERK1/2 (Thr202/Tyr204,Thr185/Tyr187),PECy5 conjugated antibodies into a centrifuge tube for a final volume of 10 µL total. This amount is good for one test.

   *Based on the number of tests/tubes being performed, it is up to the end user to adjust antibody volume amounts at similar ratios (e.g., for 10 tests, the working cocktail solution will contain 50 µL of anti-phospho-Akt (Ser473), Alexa Fluor™ 555 and 50 µL of anti-phospho-ERK1/2 (Thr202/Tyr204,Thr185/Tyr187), PECy5 for a total of 100 µL). Aliquot 10 µL of the working cocktail solution per test tube sample. This solution should be prepared as needed but if temporary storage is needed please keep in the dark at 2–8°C.

Assay Instructions

NOTE: This assay protocol has been optimized using human Jurkat cells. However, this kit is suitable for measuring the extent of Akt and ERK1/2 target-specific detection of activation via phosphorylation on a variety of human cell types. Alternate species reactivity must be confirmed by the end user.

I. Cell Culture and Stimulation (Used for example purposes)

1. Prepare cells of interest into two tissue culture flasks (treated or untreated) overnight in a 37°C incubator with 5% CO_2. Cells should be at about 90% confluent the next day.
2. For the flask labeled "treated," treat cells accordingly (e.g., chemically treated using insulin or compound of choice). The intent is to induce a signaling response (either P13K, MAPK, or both cell signaling pathways) for the given cell type. The other flask labeled "untreated," will serve as a control.
3. Incubate the flasks in a 37°C incubator with 5% CO_2. Exposure time and treatment concentrations are determined at the discretion of the end user.
4. Deactivate cells by exchanging out the growth media with fresh growth media or 1X PBS.

* All cell treatments and experimental samples are determined by the end user. This section is provided only as an example for inducing P13K or MAPK (or both) cell signaling response by measurement of phospho-Akt and phospho-ERK1/2 activation.

II. Fix and Permeabilize Cells

1. After cellular deactivation, spin down the "treated" and "untreated" testing samples at 300 xg for 5 minutes and discard the media.
2. Resuspend cells by adding 50 µL of 1X Assay Buffer per 100,000 cells (for larger cell samples, i.e., 1×10^6 cells, add 500 µL 1X Assay Buffer to cell sample).

3. Add equal parts Fixation Buffer to cell suspension (1:1). So for every 50 µL of 1X Assay Buffer per 100,000 cells, add an additional 50 µL Fixation Buffer for a total of 100 µL cell fixation solution, and mix sample by gently pipetting up and down. (Similarly, add 50 µL of Fixation Buffer for every extra 100,000 cells evaluated to keep the 1:1 ratio consistent). Incubate for 10 minutes on ice.
4. Spin down cells at 300 xg for 5 minutes in a tabletop centrifuge and discard supernatant.
5. Permeabilize cells by adding 100 µL ice-cold 1X Permeabilization Buffer per 100,000 cells and incubate on ice for 10 minutes (For larger cell samples, i.e., 1 million cells, add 1 mL ice-cold Permeabilization Buffer).
6. Spin down cells at 300 xg for 5 minutes in a tabletop centrifuge and discard supernatant.
7. Resuspend cells in 90 µL 1X Assay Buffer per 100,000 cells in a microcentrifuge tube (Compatible for the Guava® Muse® Cell Analyzer; Please see "Materials Required but Not Supplied" on page 3).

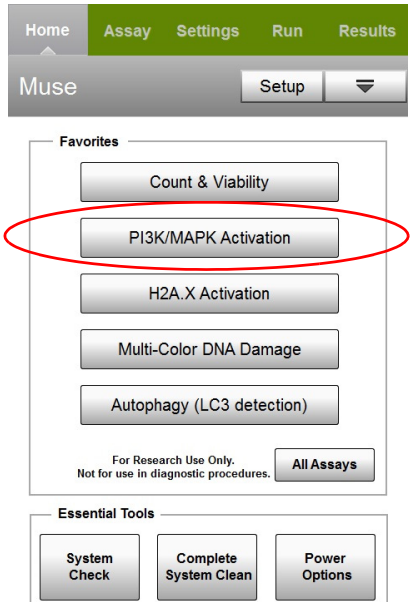
III. Cell Staining and Analysis

1. For multiplexing, add 10 µL of the antibody working cocktail solution as previously described into each microcentrifuge tube containing the cell suspension.
2. Incubate cell testing samples for 30 minutes in the dark at room temperature.
3. Following incubation step, add 100 µL of 1X Assay Buffer to each microcentrifuge testing sample and centrifuge at 300 xg for 5 minutes in a tabletop centrifuge. Discard supernatant.
4. Resuspend cells in each microcentrifuge tube with 200 µL of 1X Assay Buffer.
5. Acquire samples on the Guava® Muse® Cell Analyzer using the on-screen instructions.

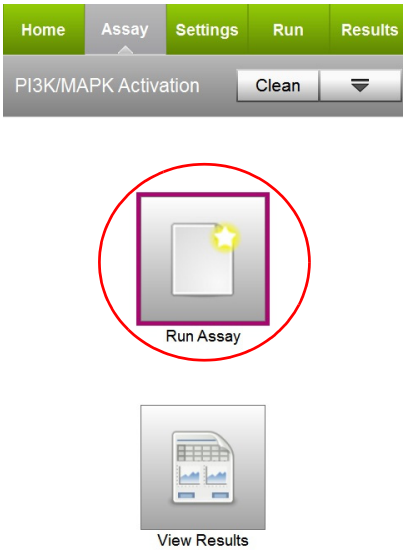
Setup and Acquisition on the Guava® Muse® Cell Analyzer

Run a System Check prior to performing the assay. For information on Muse® System Check, refer to the *Guava® Muse® Cell Analyzer User's Guide*.

1. Select **P13K/MAPK Activation** from the main menu.



2. Select **Run Assay**.

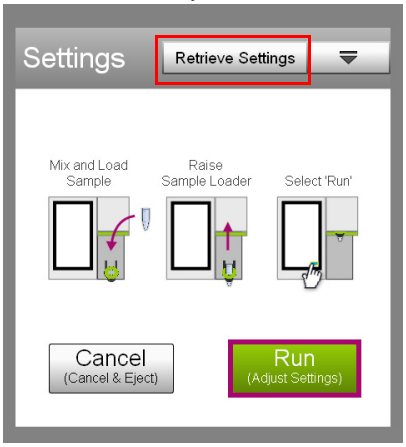


3. Adjust the instrument settings:

   - Load the sample for adjusting the settings and select **Run**.

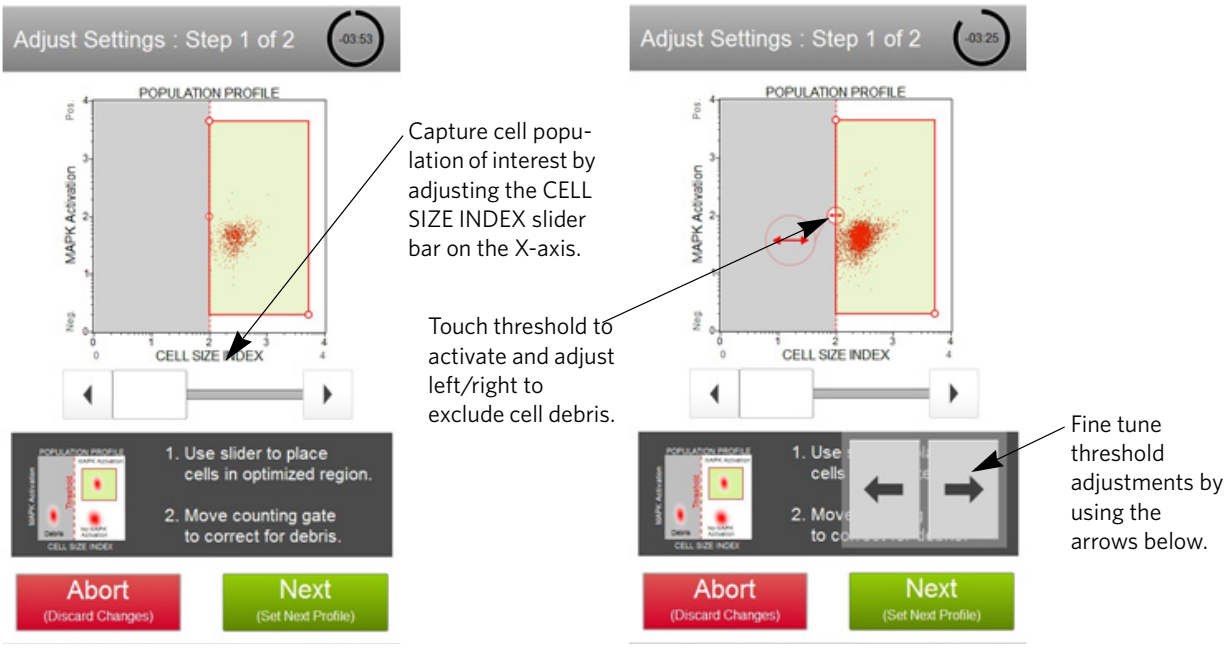
     NOTE: Perform the adjust settings step using a negative control (e.g., untreated sample), then verify the settings using a positive control (e.g., treated sample).

- Or, to retrieve previously saved instrument settings, select **Retrieve Settings**. For more information on retrieving settings, see the *Guava Muse Cell Analyzer User's Guide*.



4. Fine tune the settings for the MAPK EXPRESSION and CELL SIZE INDEX plot, if necessary.
   - Adjust the CELL SIZE INDEX slider accordingly to capture the cell population of interest (see the on-screen instructions for example).
   - Drag the threshold left or right to exclude cell debris. Drag to make large changes. Touch the arrow buttons below the plot to make small changes. The arrow buttons appear after you touch the threshold.

   NOTE: If the acquisition times out (after 4 minutes), you can select **Back** to restart the adjust settings step, or **Next** to accept the settings and continue to the next step.



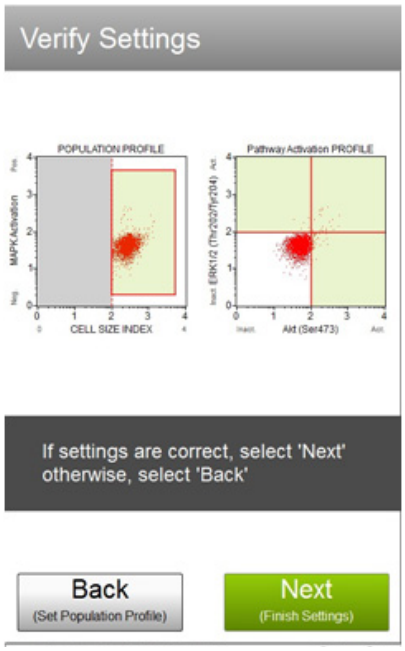
5. Select **Next** when you have completed the adjustments.
6. Fine tune the settings for the ERK1/2 (Thr202/Tyr204) vs. Akt (Ser473) plot, if necessary.
   - SETTING THE GATE: To set the quadrant marker properly, prepare a control cell sample (no stimulation) and place the cell population in the lower left (LL) quadrant of the quadrant stat. This will represent your negative cells.

- Adjust the slider bars (on the X and Y-axis) to place all populations: Negative (LL), ERK1/2 Phosphorylation (UL), Akt Phosphorylation (LR), and Dual Pathway Activation (UR) on scale. If the cell sample is not stimulated (e.g., PMA treatment) a great majority of the cell population will fall in the "Negative" (lower left) quadrant. Adjust the quadrant markers to place the cell population(s) immediately in the lower left quadrant (see diagram below).
- Adjust the quadrant markers. You can move the marker intersection in any direction, as well as adjust the angle of each line. To move the markers as they are, touch the open circle at the intersection and drag the markers to make large changes, or touch the arrow buttons below the plot to make small changes (A). To adjust the angle of either line, touch the solid circle and drag in an arc, or touch the arrow buttons below the plot (B and C).

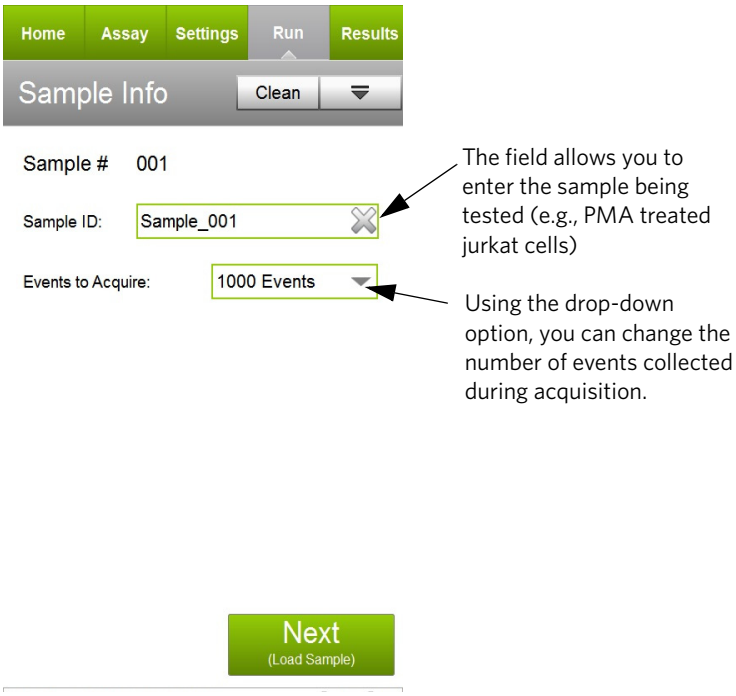
A. Moving the quadrant marker B. Adjusting the X-axis C. Adjusting the Y-axis

7. Select **Next** when the marker adjustments are complete.

8. Verify the settings. If the settings are correct, select **Next**. Otherwise, select **Back** and repeat steps 4 through 7, as necessary.



9. Enter the sample ID by touching the field, then using the keypad to input the ID. Touch **Done** when you finish entering the ID. If necessary, change the Events to Acquire by touching the field, then selecting the value from the pop-up menu. Select **Next**.

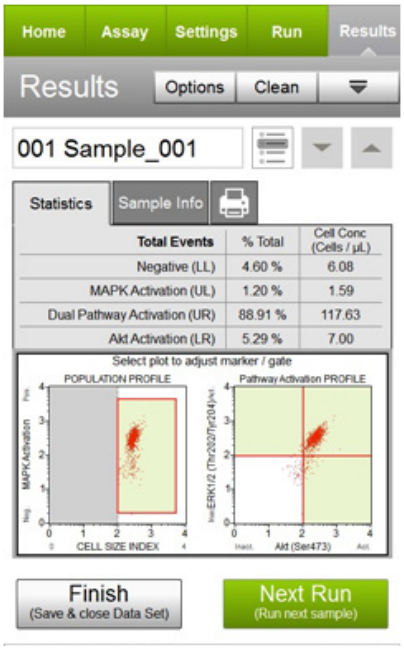


10. Follow the on-screen instructions and mix the first sample. Load the sample on the instrument loader arm. Select **Run** to acquire the sample.

During acquisition, live statistical values are generated and represented on a dot plot graph. Quantitative measurements include Negative, MAPK activation, Akt activation, and Dual pathway activation.

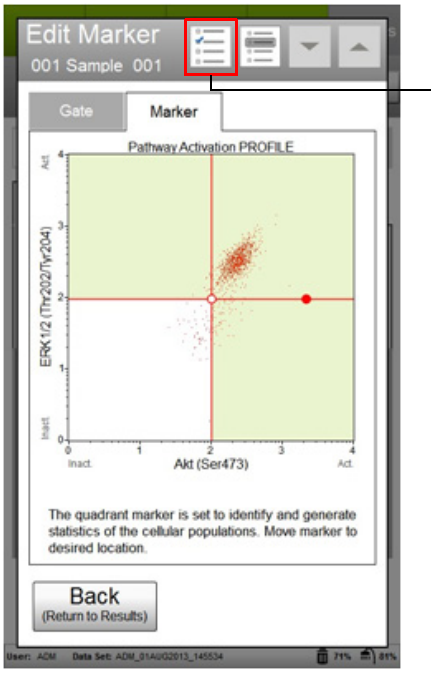
11. When acquisition is complete, the results are displayed. If desired, select **Plots** to display dot plots for the sample.

    You can view or change the sample ID, as well as add annotations for the current sample by selecting the Sample Info tab. To print the results for the current sample select the printer tab.



12. (Optional) If changes are needed to the gates assigned, touch a plot to enlarge it, then adjust the cell size gate accordingly, as described in steps 4 and 6, respectively. You cannot adjust the cell size threshold after the sample has been acquired.

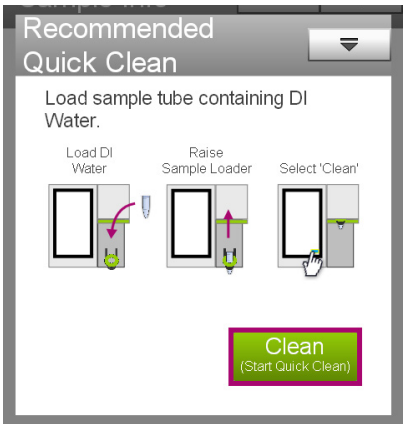
If you adjust the gate on subsequent samples and wish to apply the changes to other samples that you already acquired, select the **Apply Changes** button in the title bar. Select the samples you want to apply the changes to or choose **Select All**, then select **Apply**. The sample you originally made changes to must be selected.



Select to apply changes to other samples.

13. If no adjustments are needed, select **Next Run** and repeat steps 9 through 12 for the remaining samples.

NOTE: During the run, a message may appear prompting you to load a tube of DI water for a Quick Clean. Load the water then select **Clean** to perform the Quick Clean. Select **Next** to continue with the run. The frequency of Quick Cleans was set by your system administrator. Your administrator may also have chosen to allow you to skip the Quick Clean when the prompt appears. You can choose to perform additional Quick Cleans at any time during a run by selecting **Clean** in the title bar, then **Quick Clean** from the menu.



14. When you have acquired the last sample, select **Finish**.
15. (Optional) Select **Options** in the title bar to rename the data set, export the data set, save the current instrument settings, or view the event log. Refer to the *Guava Muse Cell Analyzer User's Guide* for more information.

Results

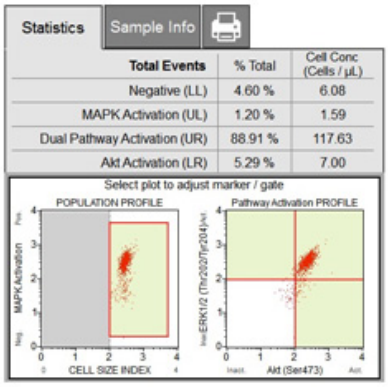
The software performs calculations and displays the data in two ways:

- A dot plot displaying cells which are negative (e.g., no pathway activation), Akt phosphorylated (PI3K pathway activation), ERK1/2 phosphorylated (MAPK pathway activation), and dual pathway activation (Akt + ERK1/2 co-activated) on quadrant plot.
- Quantitative percentage (%) measurements of the total events in a table format: Percentages of negative, Akt phosphorylated, ERK1/2 phosphorylated, and dual pathways activated.

Results from each run are stored in a data file, as well as its corresponding spreadsheet (CSV) file. The data file and spreadsheet file contain the following statistics:

- Sample Number
- Sample ID
- Percent totals for the Negative/Akt phosphorylated/ERK1/2 phosphorylated/Dual pathway activation for PI3K and MAPK

Figure 2: Jurkat cells were exposed to 100 ng/mL PMA for 5 minutes to induce a cell signaling cascade response, fixed, permeabilized, and then stained with both anti-phospho-ERK 1/2 (Thr202/Tyr204, Thr185/Tyr187) and anti-phospho Akt (Ser473) antibodies in multiplex. Samples were acquired using the Muse® Cell Analyzer and statistical results are shown above.
The statistics captured in this assay show the relative percentages for each population as it is calculated and presented in the dot plot above. Cells which express ERK1/2 can be seen by the data on the top two quadrants of the dot plot (ERK1/2 only and ERK1/2 paired with Akt, representing about 1.20% and 88.91% of the total cell population, respectively).Alternatively, cells which express Akt can be seen by the data in the two right quadrants of the dot plot (Akt only and Akt paired with ERK1/2, representing 5.29% and 88.91%, respectively). All cells that do not express either Akt or ERK1/2 (e.g., negative) can be calculated and found in the lower left quadrant, 4.60%.



Technical Tips

1. For cellular staining and analysis to be most effective, make sure that test cells have good viability prior to use.
2. For certain cell cultures cell pellets may become hazy or transparent following the fixation step, making it difficult to see. If sampling a small collection of cells for flow analysis, it is recommended that all steps be performed in a smaller collection tube (e.g., centrifuge tube).
3. Do not mix or interchange reagents from various kit lots.

4. Mix each cell sample thoroughly on a mixer before acquiring samples for consistent and accurate results. However, avoid vigorous mixing, which can cause cellular breakdown and splashing, resulting in volume loss and erroneous results.
5. The default number of events to acquire is 1000 events. You may select a different number, however, your statistical error will increase as you decrease the number of acquisition events.
6. If results deviate from expected values, prepare a freshly stained sample and reacquire the data.
7. Periodically run Quick Clean using a tube of DI water (after every 20 sample acquisitions) to prevent a buildup from cellular debris in the system. If your samples contain significant amounts of cellular debris, run the Quick Clean cycle more often to prevent clogs or blockage.
8. If you are acquiring data from a sample but the progress bar is not moving, there is probably either insufficient volume to continue to acquire the sample or a blockage of the flow system. First check to ensure that there is at least 100 µL of sample in the tube. If not, add additional buffer to bring the volume up to 100 µL or proceed to the next sample. If the sample volume is greater than 100 µL, then the lack of events is probably due to a clog. A clog or blockage can be caused by cell aggregates, cell debris, bleach crystals, or other particulates. Perform a backflush to flush out the clog into a tube containing 20% bleach. Then run Quick Clean to remove bleach residue. If this procedure does not alleviate the problem, refer to the *Guava® Muse® Cell Analyzer User's Guide* for additional troubleshooting tips, or contact Luminex Technical Support for help.
9. The Muse PI3K/MAPK Dual Pathway Activation Kit works best with samples in single cell suspension. Cell aggregates may clog or be excluded from the flow cell, affecting the accuracy of the results. If you wish to use the Muse PI3K/MAPK Dual Pathway Activation Kit with a "clumpy" cell line, it is recommended to order Muse Cell Dispersal Reagent (Catalog No. MCH100107) to disaggregate the cells. Contact customer service or visit our website at www.luminexcorp.com for detailed information on the Muse Cell Dispersal Reagent and assay method. For more troubleshooting tips refer to the *Guava Muse Cell Analyzer User's Guide*.

Troubleshooting

Potential Problem	Experimental Suggestions
Acquisition taking longer than expected or progress bar stops during acquisition	Ensure that the System Check procedure was run and passed. If the progress bar stops during acquisition, the fluid system may be clogged. Run a Quick Clean procedure to clean the capillary. It can be performed during or after an assay.
Instrument clogging	If the instrument is clogged, run a Quick Clean procedure to clean the capillary. This procedure can be performed at any time during an assay between samples.
No detectable phosphorylation / activation in testing samples	Since phospho-specific activation can be very quick, transient responses, in order to capture this phosphorylation event samples must be immediately fixed to "freeze" the given activation state in time.
Low Cell Concentration warning during acquisition	Ensure that cells are counted properly prior to beginning the experiment. The assay instructions are optimized to give you a range of cells between 300-700 cells/µL in the final sample volume so accurate population count results are obtained. A substantial decrease in cell numbers can lead to difficulty in adjusting settings.

Potential Problem	Experimental Suggestions
High Cell Concentration warning during acquisition	If the concentration of the stained cell sample is high (>1200 cells/µL), dilute the sample further with Muse® Cell Cycle Reagent to adjust the cell concentration between 300 and 700 cells/µL.
High %Cvs (wide peaks) or false peak	Although the assay procedure has been optimized to function for multiple cells types, every cell line behaves differently. The wide peaks or false peak may indicate that: • The sample is poorly fixed and stained as a result of cell aggregates. Ensure your sample is a single-cell suspension before fixing and staining. • Cell concentration is too high. Decrease the number of cells by diluting the sample to 300–700 cells/µL. The Guava® Muse Cell Analyzer gives the most accurate data when the flow rate is between 300 and 700 cells/µL.
Low level of staining	• Although the assay procedure has been optimized to function utilizing multiple cell types, every cell line behaves differently. A low signal may indicate that the cells need to be stained at a higher volume. • Verify that the System Check procedure was performed and the results passed.
Variability in day-to-day experiments	• If the results are inconsistent, check that the samples were well mixed prior to acquisition. Cells may quickly settle in your samples and your results will be inaccurate unless the cells are mixed just prior to acquisition. • Monitor experimental cell cultures to ensure that cell viability and cell numbers being analyzed are consistent. Any drop in cell numbers or viability can influence experimental results. • If there appears to be day-to-day variation of the staining pattern, ensure the Guava Muse Cell Analyzer is working properly. Check the Muse System Check log to ensure day-to-day instrument variation is low.

References

1. Krutzik PO, et al. Intracellular phospho-protein staining techniques for flow cytometry: monitoring single cell signaling events. *Cytometry A*. 2003;55(2):61–70.
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Related Products

- Muse® H2A.X Activation Dual Detection Kit – Catalog No. MCH200101
- Muse® EGFR-RTK Activation Dual Detection Kit – Catalog No. MCH200102
- Muse® PI3K Activation Dual Detection Kit – Catalog No. MCH200103
- Muse® MAPK Activation Dual Detection Kit – Catalog No. MCH200104
- Muse® Bcl-2 Activation Dual Detection Kit – Catalog No. MCH200105
- Muse® Multi-Color DNA Damage Kit – Catalog No. MCH200107
- Muse® System Check Kit – Catalog No. MCH100101
- Muse® Count & Viability Kit (40mL) – Catalog No. MCH100102
- Muse® Annexin V & Dead Cell Kit – Catalog No. MCH100105
- Muse® Cell Dispersal Reagent – Catalog No. MCH100107

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