



# Muse<sup>®</sup> Ki67 Proliferation Kit User's Guide

For Research Use Only. Not for use in diagnostic procedures.  
4600-3460, Rev C  
Catalog No. MCH100114 (100 tests)  
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# Application

The Muse® Ki67 Proliferation Assay allows for the determination of the percentages of proliferating and non-proliferating cells based on Ki67 expression. The software provides percentages of both Ki67(+) cells and Ki67(-) cells.

The Muse Ki67 Proliferation Assay is for use with the Guava® Muse® Cell Analyzer. The Muse System makes sophisticated fluorescent-based analysis fast, easy, convenient, and affordable. After loading samples onto the Muse Cell Analyzer, the intuitive software provides detailed or summary analysis of your cell sample in a few short steps.

Sufficient reagent is provided for the preparation and analysis of 100 tests.

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# Test Principle

The assessment of proliferation, or cell growth, is a critical aspect of cellular research. Several markers are expressed during cell proliferation, including the nuclear antigen Ki67, which is tightly associated with proliferation.<sup>1-3</sup> Ki67 is a prototypic cell cycle-related nuclear protein, expressed by proliferating cells in all phases of the active cell cycle (G1, S, G2, and M phases), but is absent in the resting G0 phase. This characteristic makes Ki67 a good marker for the identification of proliferating cells.

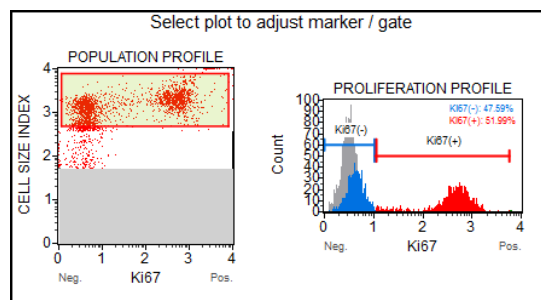
Changes in Ki67 expression has found widespread applications in cell biology, immunology, and drug discovery research. Ki67 expression has thus been used in evaluating potency of growth factors and compounds that alter cell cycle, in evaluating the anti-proliferative activity of drugs, and in the study of cytotoxicity.<sup>3-7</sup> Characterization of Ki67 expression has also been studied in research on the mechanism of several cancers such as breast cancer, ovarian tumors and prostate cancers.<sup>8-12</sup>

The Muse® Ki67 Proliferation Assay utilizes Ki67 expression to identify proliferating cells and distinguish them from non-proliferating cells. Two populations of cells can be distinguished in this assay:

- Ki67(-) Cells: cells that are not proliferating or stained with IgG1
- Ki67(+) Cells: cells that are proliferating

Each plot has moveable markers. The first plot has a threshold marker, allowing you to eliminate debris based on size, as well as a gate, allowing you to gate on cells. The second plot has two histogram markers, allowing you to obtain statistics on two cell populations—Ki67(-) and Ki67(+) and overlay the positive sample date and negative (isotype) data.

**Figure 1:** Representative plots from the Muse Ki67 Assay. The first plot has a threshold marker, allowing you to eliminate debris based on cell size, as well as a gate. The second plot shows overlaid histograms providing data on Ki67 expression.



# Summary of Protocol

Culture cells, including for positive and negative controls:

Wash cell samples once with PBS, then transfer  $5.0 \times 10^3$  to  $1.0 \times 10^5$  cells/sample into each tube/well.

Prepare 1X Fixative Solution (50  $\mu$ L/test) and 1X Assay Buffer (500  $\mu$ L/test).

Add 50  $\mu$ L of 1X Fixation Solution to each tube/well. Mix and incubate for 15 minutes at room temperature.

Add 150  $\mu$ L of 1X Assay Buffer, centrifuge, and remove supernatant.

Add 100  $\mu$ L of Permeabilization Solution to each tube/well. Mix and incubate for 15 minutes at room temperature.

Add 100  $\mu$ L of 1X Assay Buffer, centrifuge, and remove supernatant.

Add 50  $\mu$ L of 1X Assay Buffer to each tube/well. Mix and incubate for 15 minutes at room temperature.

Add 10  $\mu$ L of either Muse® Hu IgG1-PE or Muse® Hu Ki67-PE to each tube/well. Mix and incubate for 30 minutes at room temperature.

Add 150  $\mu$ L of 1X Assay Buffer to each tube/well and run on the Muse® Cell Analyzer.



## Kit Components

- Muse® Hu Ki67 Antibody (Part No. 4700-1667, 100 tests/vial)
- Muse® Hu IgG1 Control (Part No. 4700-1669, 100 tests/vial)
- 5X Assay Buffer (Part No. CS202124, 50 mL/vial)
- 5X Fixation Buffer (Part No. 4300-0250, two 3-mL vials)
- Permeabilization Buffer (Part No. CS202125, 10 mL/vial)

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## Materials Required but Not Supplied

- Guava® Muse® Cell Analyzer
- Cellular samples
- Micropipettors
- Disposable micropipettor tips
- Microcentrifuge tubes with screw caps, 1.5 mL (VWR Catalog No. 16466-030, or equivalent)
- Vortex mixer
- 100% bleach solution
- Disposable gloves
- Deionized water
- 1X PBS, calcium and magnesium free
- Tabletop centrifuge
- 96-well plate, optional
- Muse® System Check Kit (Catalog No. MCH100101)
- Guava ICF Instrument Cleaning Fluid (Catalog No. 4200-0140), optional

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## Precautions

- The Muse® Ki67 Proliferation Kit is intended for research use only.
- Wear proper laboratory attire (lab coat, gloves, safety glasses) when handling this reagent.
- Muse Hu Ki67 Antibody, Muse Hu IgG1 Control, 5X Assay Buffer, and Permeabilization Buffer contain sodium azide, which is toxic if ingested. Reagents containing sodium azide should be considered a poison. If products containing sodium azide are swallowed, seek medical advice immediately and show product container or label. (Refer to NIOSH, National Institute for Occupational Safety and Health; CAS#: 2628-22-8; and also to GHS, the Globally Harmonized System of Classification and Labeling of Chemicals.) Aqueous solutions of sodium azide, when mixed with acids, may liberate toxic gas. Any reagents containing sodium azide should be evaluated for proper disposal. Sodium azide may react with lead and copper plumbing to form highly explosive metal azides. Upon disposal, flush with large volumes of water to prevent build-up in plumbing. Check with regulatory agencies to determine at what concentration sodium azide may cause a product to be regulated as hazardous.
- The fluorochrome-conjugated antibodies in this reagent are light-sensitive and must be stored in the dark at 2 to 8°C.
- Avoid microbial contamination of the solution, which may cause erroneous results.
- All biological specimens and materials should be handled as if capable of transmitting infection and disposed of with proper precautions in accordance with federal, state, and local regulations. Never pipette by mouth. Avoid specimen contact with skin and mucous membranes.
- Exercise care to avoid cross contamination of samples during all steps of this procedure, as this may lead to erroneous result.

- 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.
- During storage and shipment, small volumes of product may become entrapped in the seal of the product vial. For maximum recovery of the product, centrifuge the vial briefly prior to removing the cap.
- Do not use the reagent beyond the expiration date.
- Safety Data Sheets (SDSs) for kit reagents are available from our website ([www.luminexcorp.com](http://www.luminexcorp.com)) or by contacting Luminex Technical Support.

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## Storage

- Store the following at 2° to 8°C:
  - Muse® Hu Ki67-PE Antibody
  - Muse® Hu IgG1-PE Antibody
  - Permeabilization Buffer
  - 5X Assay Buffer
- Store the 5X Fixation Solution at room temperature (18° to 25°C).



**CAUTION:** Fluorochrome-conjugated antibodies should always be stored at 2° to 8°C. Any deviation in temperature for long periods of time may compromise the performance of the antibodies.

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## Before You Begin

Luminex recommends that the cell samples be run shortly after the sample preparation has been completed. While some cell types have been shown to yield stable results for up to 24 hours when stored capped at 2 to 8°C after staining with the Muse® Ki67 Proliferation Kit, the stability of individual cell types may vary. Optimal staining is obtained when cell concentrations are between  $5 \times 10^3$  and  $1 \times 10^5$  cells/mL.

**Time considerations:** The process of staining cells with the Muse Ki67 Proliferation Kit using the recommended protocol below takes 2 hours. Acquiring your data on your Guava® Muse Cell Analyzer takes less than 3 minutes per sample, depending on the concentration and desired number of events to acquire. However, preparing your cells for testing may require periodic maintenance and cultivation. Once you cultivate the proper number of cells for the experiment, it can take between 2 hours and 5 days of stimulation for proliferation effects.

**NOTE:** For details on how to culture and prepare cell samples, including positive and negative control samples, see "Appendix A: Cell Sample Preparation" on page 14.

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

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# Reagent Preparation

## 1X Assay Buffer

The 1X Assay Buffer is supplied as a 5X concentrate. It must be diluted to 1X with DI water prior to use. Approximately 500  $\mu\text{L}$  of 1X Assay Buffer is required per sample to be stained.

1. Warm the 5X Assay Buffer to room temperature to completely dissolve any crystals that may have formed during storage.
2. Mix 1 part 5X Assay Buffer with 4 parts DI water.
3. Gently vortex to mix.

## 1X Fixation Buffer

The 1X Fixation Buffer is supplied as a 5X concentrate. It must be diluted to 1X with 1X PBS prior to use. Approximately 50  $\mu\text{L}$  of 1X Fixation Buffer is required per sample to be stained.

1. Warm the 5X Fixation Buffer to room temperature to completely dissolve any crystals that may have formed during storage.
2. Mix 1 part 5X Fixation Buffer with 4 parts 1X PBS.
3. Gently vortex to mix.

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# Example Cell Staining Protocol

1. Add 200  $\mu\text{L}$  of cells in suspension to each tube/well. For instructions on preparing cell suspensions, see "Appendix A: Cell Sample Preparation" on page 14. Make sure to stain positive and negative controls.
2. Centrifuge the cells for 5 minutes at 300 x g.
3. Aspirate off the supernatant.
4. Resuspend the cells in 200  $\mu\text{L}$  1X PBS.
5. Gently vortex the cells to disperse the pellet.
6. Centrifuge the cells for 5 minutes at 300 x g.
7. Aspirate the supernatant.
8. Add 50  $\mu\text{L}$  of the 1X Fixation Solution to each sample and mix thoroughly.
9. Incubate at room temperature for 15 minutes.
10. Wash samples once in 1X Assay Buffer.
  - a. Add 150  $\mu\text{L}$  of the 1X Assay Buffer to each sample and mix thoroughly.
  - b. Centrifuge for at least 5 minutes at 300 x g.
  - c. Aspirate off the supernatant.
11. Add 100  $\mu\text{L}$  of Permeabilization Buffer to each sample and mix thoroughly.
12. Incubate at room temperature for 15 minutes.

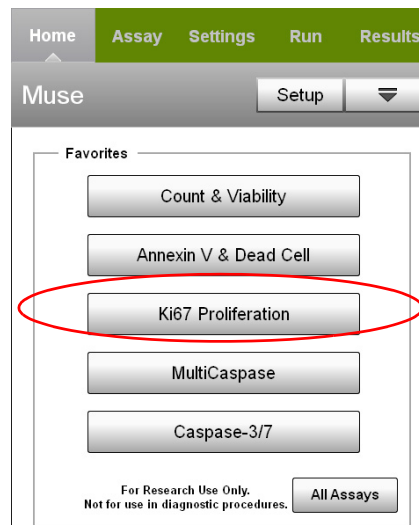
13. Wash samples once in 1X Assay Buffer.
  - a. Add 100 µL of the 1X Assay Buffer to each sample and mix thoroughly.
  - b. Centrifuge for at least 5 minutes at 300 x g.
  - c. Aspirate off the supernatant.
14. Add 50 µL of 1X Assay Buffer to each sample mix thoroughly.
15. Incubate at room temperature for 15 minutes.
16. Add 10 µL of either the Muse® Hu IgG1-PE or Muse Hu Ki67-PE to each sample and mix thoroughly.
17. Incubate the samples at room temperature for 30 minutes in the dark.
18. After incubation, add 150 µL of 1X Assay Buffer to each sample.

Samples are ready to run on the Guava® Muse Cell Analyzer.

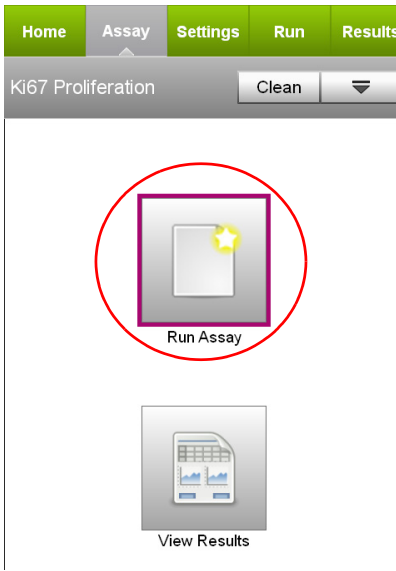
## Setup and Acquisition on the Guava® Muse® Cell Analyzer

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 **Ki67 Proliferation** from the main menu.

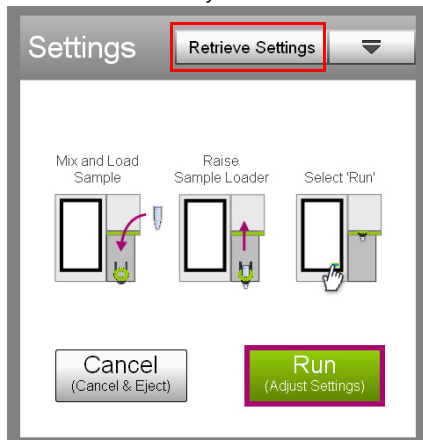


2. Select **Run Assay**.



3. Adjust the instrument settings.

- Load the IgG1 isotype control for adjusting the settings and select **Run**.
- 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*.

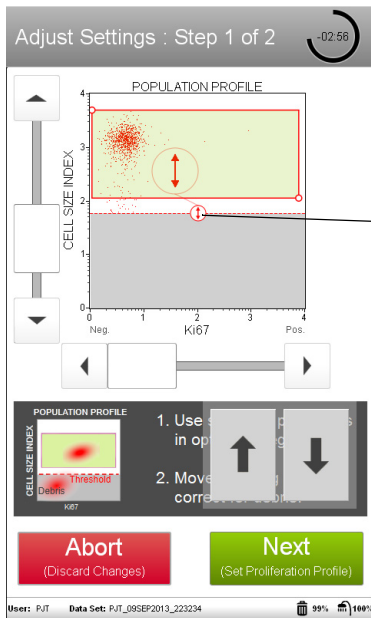


4. Fine tune the settings for the Ki67 vs. CELL SIZE INDEX plot, if necessary.

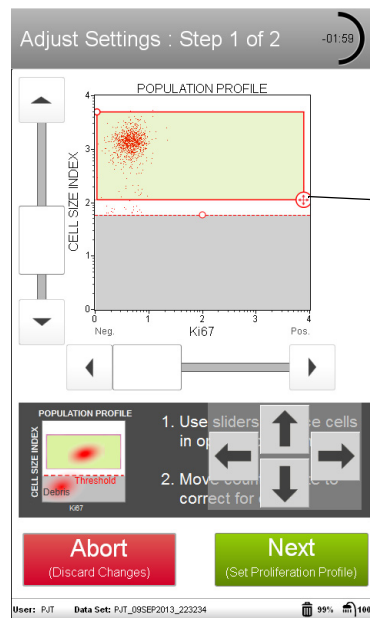
- Adjust the CELL SIZE INDEX slider to the left of the plot to move the cell population into the gate (green region).
- Drag the threshold to exclude any cellular debris. Touch the threshold and drag to make large changes. Touch the arrow buttons located below the plot to make small changes. The arrow buttons appear after you touch the threshold.
- Drag the upper-left or lower-right corner of the gate to encompass the cell population.



**NOTE:** If the adjust settings step times out (after 2 minutes), remove the tube and mix well before reloading. Then, select **Back** to restart the adjust settings step. Or, select **Next** to accept the settings and continue to the next step.



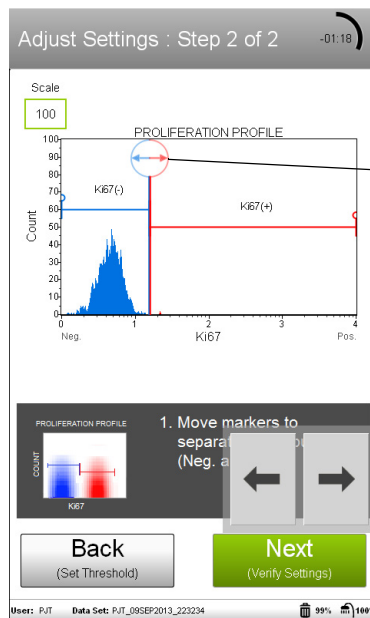
Touch threshold to move up/down.



Touch and drag upper-left or lower-right corner to adjust the gate.

This example data shows typical gate and marker settings. The threshold was raised to remove debris.

5. Select **Next** when the adjustments are complete.
6. Fine tune the markers on the Ki67 histogram plot, if necessary.
  - Touch the Scale at the top of the y-axis to change the count.
  - Adjust the histogram markers, if necessary. To move the markers, touch the open circles at the top of each end of the markers. Drag the markers left/right to make large changes, or touch the arrow buttons below the plot to make small changes.



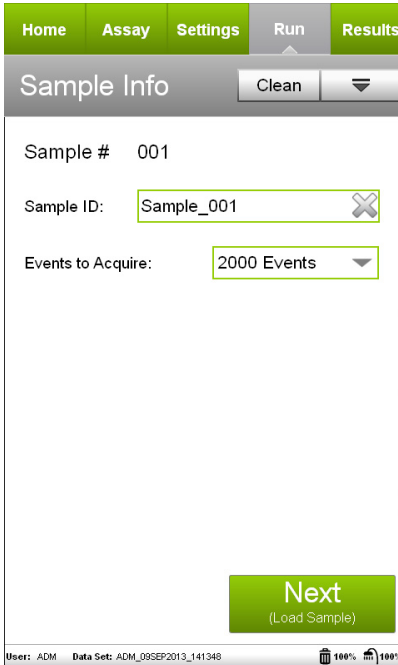
Touch open circles to move the markers left/right.

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

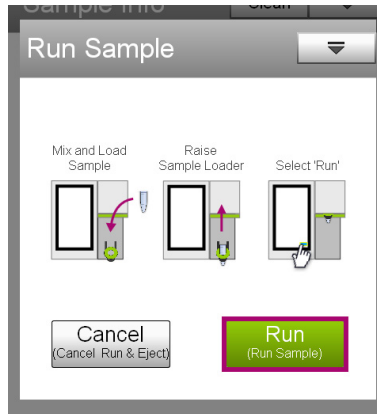
- Verify the settings for the isotype control sample. Then select **Back** and repeat steps 4 through 7 using a positive control. When the settings are correct, select **Next**.



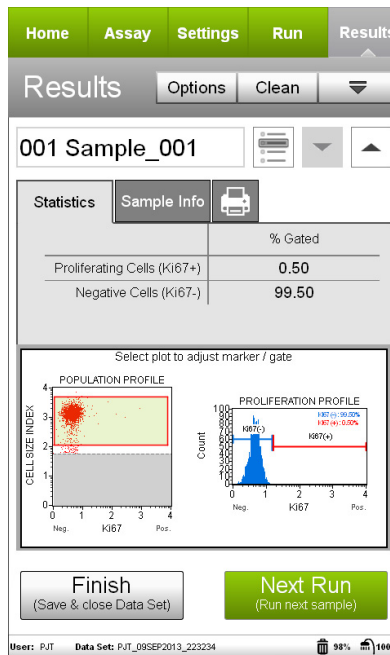
- Enter the sample ID by touching the field, then using the keypad to input the ID. Touch **Done** when you finish entering the ID. If necessary, change the Events to Acquire by touching the field, then selecting the value from the pop-up menu. Select **Next**.



- Mix the first sample and load it on the instrument. Select **Run** to run the sample.




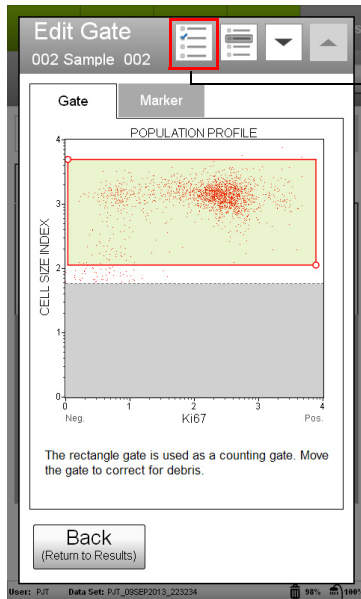
- When acquisition is complete, the results are displayed. You can view or change the sample ID, as well as add annotations for the current sample by selecting the Sample Info tab. To print the results for the current sample select the printer tab.



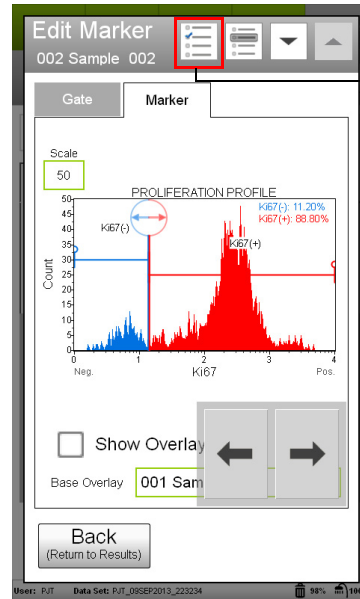
- (Optional) If changes are needed to the gate or markers, touch a plot to enlarge it, then adjust the gate and/or marker as described in steps 4 and 6, respectively. You cannot adjust the cell size threshold after the sample has been acquired.

If you wish to overlay histogram data from another sample, for example, the isotype control, select the histogram plot, then select **Show Overlay**. Select the base overlay sample from the Base Overlay field. The default is the first sample. The base overlay histogram appears in grey behind the current sample histogram.

If you adjust the gate or markers 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.



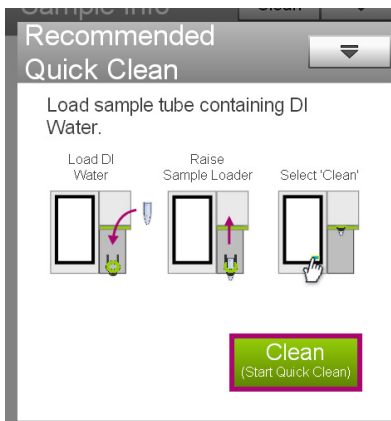
Apply changes to other samples.



Select to apply changes to other samples.

- 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.



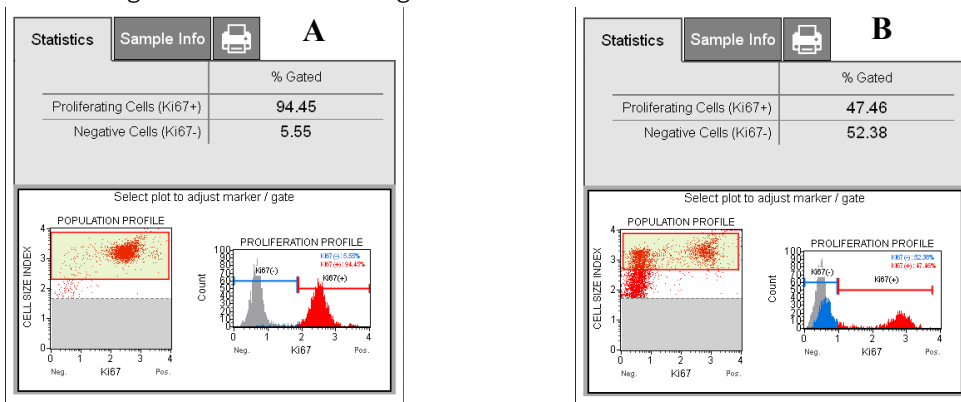
- When you have acquired the last sample, select **Finish**, enter a name for the data set or leave the default, and select **OK**.
- (Optional) Select **Options** in the title bar to rename the data set, export the data set, save the current instrument settings, or view the event log. Refer to the *Guava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

## Results

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
- cell concentration (cells/ $\mu$ L)
- percentage of Ki67 proliferating and negative cells:
  - left (blue): Ki67 (-)
  - right (red): Ki67 (+)

**Figures A and B:** Results obtained with the Muse® Ki67 Cell Proliferation Kit. Figure A shows results for healthy proliferating Jurkat cells (red) with an optional histogram overlay of the isotype control (grey). Figure B shows human PBMC cells stimulated with PHA (red and blue) with an optional histogram overlay of the isotype control (grey). The statistics show population percentages in the stained sample. The dot plot shows Ki67 staining vs. Cell Size Index and the histogram shows Ki67 staining.



## Technical Tips

1. 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.
2. The default number of events to acquire is 2000. You may select a different number; however, your statistical error will increase as you decrease the number of acquisition events.
3. If results deviate from expected values, prepare a freshly stained sample and reacquire the data.
4. 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.
5. If you are acquiring 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  $\mu$ L of sample in the tube. If not, add additional buffer to bring the volume up to 100  $\mu$ L or proceed to the next sample. If the sample volume is greater than 100  $\mu$ L, 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 Technical Support for help.

Muse Ki67 Proliferation Kit works best with samples in a homogeneous, single cell suspension. Cell aggregates may clog or be excluded from the flow cell, affecting the accuracy of your results.

## Troubleshooting

| Potential Problem  | Experimental Suggestions  |
|--|---|
| Acquisition taking longer than expected or progress bar stops during acquisition | Ensure that the Muse® System Check (Part No. MCH100101) procedure was run and passed. If the progress bar stops during acquisition, the fluid system may be clogged. Run a Quick Clean procedure. It can be performed during or after an assay. This will wash away any material forming within the glass capillary walls.  |
| Instrument clogging; too many cells  | <ul style="list-style-type: none"> <li>Cell concentration too high: Optimal cell concentration is <math>2.5 \times 10^4</math> to <math>5 \times 10^5</math> cells/mL (<math>5 \times 10^3</math> to <math>1 \times 10^5</math> cells/sample). If the cell concentration is outside this range, dilute the sample.</li> <li>Run a Quick Clean to clean out the capillary. This procedure can be performed during or after an assay. This will wash away any material forming within the glass capillary walls.</li> </ul> |
| Low Cell Concentration warning during acquisition                                | The sample concentration may be too low. The assay instructions are optimized to give you a range of cells between 100–500 cells/ $\mu$ L in the final sample volume so accurate population count results are obtained. Repeat sample preparation with a lower dilution factor to allow for adequate cell numbers. A substantial decrease in cell numbers can lead to difficulty in adjusting settings.   |
| High Cell Concentration warning during acquisition                               | If the concentration of the stained cell sample for acquisition is high ( $>500$ cells/ $\mu$ L), the accuracy of data will most likely be compromised. Dilute the sample further with 1X Assay Buffer to adjust the cell concentration below 500 cells/ $\mu$ L. For best results, we recommend that the cell concentration is in the range of 200 to 300 cells/ $\mu$ L.  |
| Background staining and/or non-specific staining of cells                        | If control samples (isotype, positive, and/or negative) appear to have large amounts of Ki67 expression, your cultures may be compromised. It is important to run negative control samples for each experiment. The negative control should be a sample from your cell culture, not treated to induce or inhibit proliferation. However, sub-optimal culture conditions may stress cells in culture, causing the antibody to non-specifically bind.   |

| Potential Problem                                      | Experimental Suggestions   |
|--|--|
| Low level of staining                                  | <ul style="list-style-type: none"> <li>To determine optimal proliferation affects, conduct a time-course study to achieve the best results for Ki67 staining.</li> <li>If there are no Ki67-positive cells, your cells may not be proliferating. If using cells that are naturally resting, ensure that a positive control (PMA, PHA, ionomycin, etc) is used. If using cells that are naturally proliferating, ensure that cultures are growing in log phase growth to maximize signal.</li> </ul>  |
| Poor separation of proliferating and non proliferating | If the separation between populations is poor, the Ki67 concentration may be too low. Muse Ki67 Proliferation Reagent has been formulated for optimal performance using the following cells: Jurkat, HeLa, PC3, HEK, MCF7, and PBMCs. Other cells may show different patterns of reactivity that require adjustments to the amount of reagent used. For best results, titer the Ki67-PE and IgG1-PE reagents to determine the optimal amount for maximal staining of cells.  |
| Variability in day-to-day experiments                  | <ul style="list-style-type: none"> <li>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.</li> <li>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.</li> <li>If there appears to be day-to-day variation of the staining pattern, ensure the Guava® Muse Cell Analyzer is working properly. Run the Muse System Check (Part No. MCH100101) procedure to verify proper instrument function and accuracy.</li> </ul> |

## Limitations

- The results of the assay are dependent upon proper handling of samples, reagents, and instruments.
- Cell types vary in the Ki67 expression optimal Ki67 staining may differ for each cell line.
- The Guava® Muse® Cell Analyzer and Muse Ki67 Proliferation Kit yield optimal results when the stained cell sample used for acquisition is between  $1 \times 10^4$  to  $5 \times 10^5$  cells/mL. To obtain the most accurate results, adjust the cell concentrations to within the recommended range. However, to optimize throughput, we recommend using between  $1 \times 10^5$  to  $5 \times 10^5$  cells/mL when possible.

## Appendix A: Cell Sample Preparation

The following protocols describe how to harvest non-adherent and adherent cells cultured in 96-well plates or in flasks or other tissue culture vessels.

## Preparing Non-adherent Cells

1. Set up initial culture conditions, such that after culture, treatment, and the removal of the inducing medium, cells are resuspended at a concentration of  $2.5 \times 10^4$  to  $5 \times 10^5$  cells/mL ( $5 \times 10^3$  to  $1 \times 10^5$  cells/sample) in complete growth medium.
2. Proceed to “Example Cell Staining Protocol” on page 5.

## Preparing Adherent Cells

For harvesting adherent cells, use your method of removal. Reagents such as EDTA or trypsin can be used to dissociate the cells from the flask and should create single-cell suspensions. If using mechanical means to dislodge the cells, Muse® Cell Dispersal Reagent (Catalog No. MCH100107) may be used to dissociate clumps.

1. Using your preferred method for dissociation, detach the cells from their culture vessel.
2. Resuspend the cells in complete growth medium so that the final concentration is between  $2.5 \times 10^4$  to  $5 \times 10^5$  cells/mL ( $5 \times 10^3$  to  $1 \times 10^5$  cells/sample).
3. Proceed to “Example Cell Staining Protocol” on page 5.

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## References

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## Related Kits

- Muse® System Check Kit - MCH100101
- Muse® Count & Viability Kit (100T) - MCH100102
- Muse® Annexin V & Dead Cell Kit - MCH100105
- Muse® Human Lymphocyte CD69 Kit - MIM100104
- Muse® Human Lymphocyte CD25 Kit - MIM100105

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