Muse® Multi-Color DNA Damage Kit User's Guide

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Introduction

The Muse® Multi-Color DNA Damage kit is designed to enable researchers a quick and easy way to detect the activation of ATM and H2A.X using the Guava® Muse Cell Analyzer. The kit was designed and optimized using a DNA damaging reagent, Etoposide, on HeLa cells as a model system. But the kit can be used with other human cell lines to investigate both the physical and chemical factors which can induce the DNA damage response through the ATM dependent signaling pathway. 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 DNA damage)
- Percentage of ATM activated cells
- Percentage of H2A.X activated cells
- Percentage of DNA double-strand breaks (dual activation of both ATM and H2A.X)

The signaling pathways by which cells respond to DNA damage is an important area of study for cancer research. The mammalian cell response to DNA damage is made up of highly coordinated signaling pathways that can initiate cell cycle arrest and repair or initiate apoptosis, depending on the extent and type of the damage. Cells that are defective in DNA damage signaling pathways can cause cancer because they lack the ability to sense and repair the damage, leading to genetic instability and ultimately uncontrolled cell growth.

The main kinase activated in response to double-stranded DNA breaks is ATM or Ataxiatelangiectasia mutated kinase. ATM is a member of the phospho inositide 3-kinase (PI3K)-related Ser/Thr protein kinase family. Inactive ATM exists as a dimer but quickly dissociates and becomes phosphorylated on Serine 1981 in response to ionizing radiation [1].

Once activated, ATM phosphorylates a number of downstream factors, including P53, CHK2, SMC1, NBS1, and Histone H2A.X [2,3]. Phosphorylation of the histone variant, H2A.X, at serine 139 by ATM is an important indicator of DNA damage [4]. As the level of DNA damage increases, the level of phospho Histone H2A.X (also known as γH2AX) increases and accumulates at the sites of DNA damage and is often used to indicate the level of DNA damage present within the cell [5]. γH2AX is also responsible for recruiting response proteins to the site of DNA damage [6].

In all, a comprehensive understanding of both ATM and Histone H2A.X activities, and understanding the consequence of its activation can provide the researcher with useful information which will be important in understanding the nature of the DNA damage response. The Muse Multi-Color DNA Damage Kit is designed to allow the researcher to monitor and accurately measure phospho-specific activation for both ATM and Histone H2A.X in a population of cells.

The Muse Multi-Color DNA Damage 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 DNA damage signaling analysis.

Product Overview

The Muse® Multi-Color DNA Damage Kit includes two directly conjugated antibodies, a phospho-specific ATM (Ser1981)-PE and a phospho-specific Histone H2A.X-PECy5 conjugated antibody to measure the extent of DNA damage in testing samples. This two color kit is designed to detect the phosphorylation state of ATM and Histone H2A.X simultaneously by flow analysis. Together, two color analysis of ATM and Histone H2A.X activation in mul-

tiplex provides a more reliable detection method for assessing the DNA damage signaling pathway. This antibody pair provides a sensitive and valuable tool to study the factors that induce DNA damage and/or affect DNA repair, and allows one to explore the linkage between DNA damage, cell cycle checkpoints, and initiation of apoptosis.

Ionizing radiation (IR) and many chemotherapeutic agents like etoposide kill cancer cells by induction of DNA DSBs. Several reports show that the levels of γ-H2A.X as detected by flow cytometry correlates with the number of DNA strand breaks, to the level of cell death and radiosensitivity [7]. Both ATM and H2A.X phosphorylate in response to DNA damaging reagents (e.g. Etoposide) or UV light, and this dual activation clearly indicates that DSBs have occurred [4,9]. Understanding when DSBs take place can help researchers understand the mechanisms involved in DNA repair and the DNA damage response.

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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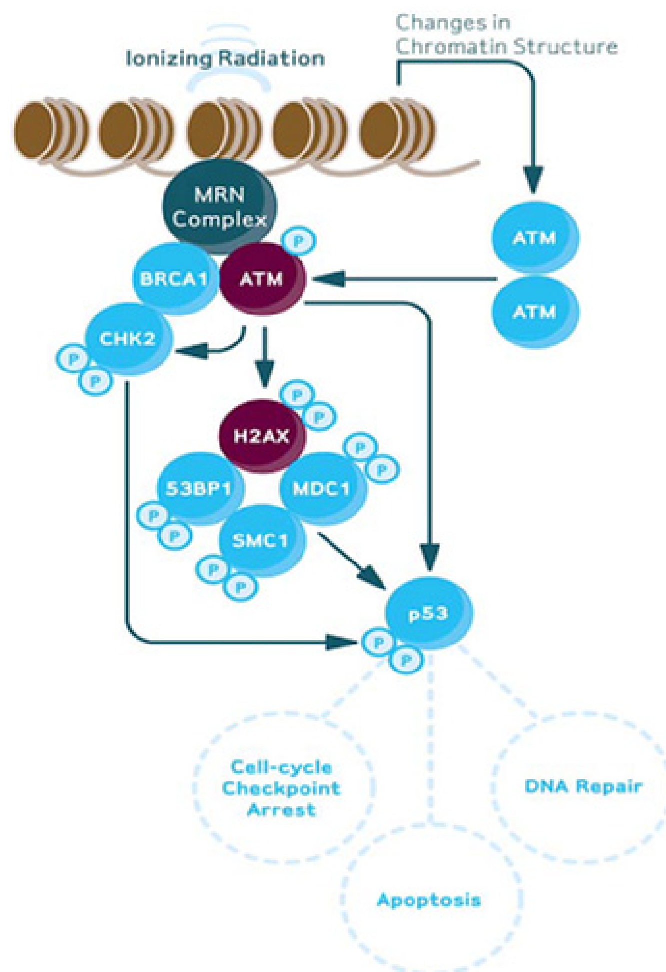
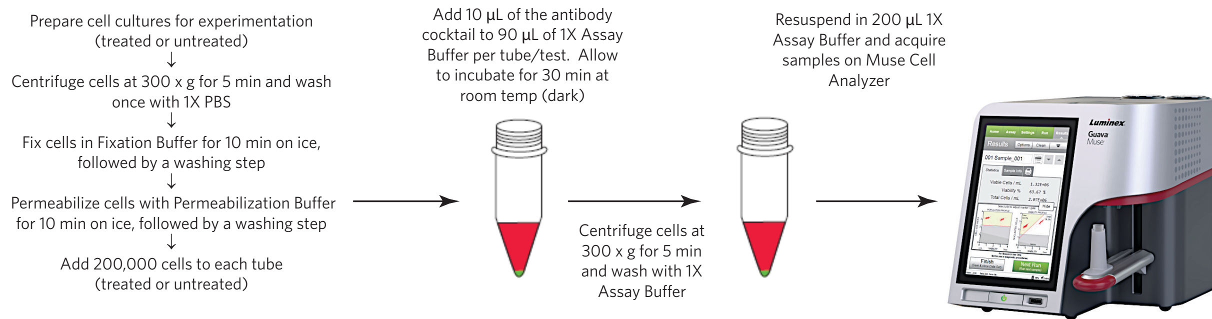
The DNA Damage Signaling Pathway



Summary of Protocol



Materials Provided

- 20X Anti-phospho-ATM (Ser1981), PE: (Part No. CS208162). One vial containing 250 µL
- 20X Anti-phospho-Histone H2A.X (Ser139), PECy5: (Part No. CS208174). 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 Ca2+ or Mg2+, 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 x g
- 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 SDS sheet for specific information on hazardous materials (SDS forms can be found on the web page or by contacting Luminex technical services).
- 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 and Stability

All reagents must be stored at 2 - 8°C.

All kit components are stable up to four (4) months from date of receipt if stored and handled correctly. **Please avoid repeated changes in temperature as this will affect the integrity of the product.**

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 perform 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 one year. Store at 2 - 8°C
2. **Antibody Working Cocktail Solution**
   The kit contains two (2) 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-ATM (Ser1981), PE and 5 µL of anti-phospho-Histone H2A.X (Ser139),PECy5 conjugated antibodies into a centrifuge tube for a final volume of 10 µL total. This amount is good for one (1) 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-Histone H2A.X (Ser139) and 50 µL of anti-phospho-ATM (Ser1981) 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 HeLa cells. However, this kit is suitable for measuring the extent of both ATM and H2A.X 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 topoisomerase inhibitors or compound of choice, or physically treated by exposure to UV irradiation). The intent is to induce DNA damage 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 a DNA damage response for measurement of phospho-ATM and phospho-Histone H2A.X activation.

II. Fix and Permeabilize Cells

5. After cellular deactivation, spin down the "treated" and "untreated" testing samples at 300 x g for 5 minutes and discard the media.
6. Resuspend cells by adding 50 µL of 1X Assay Buffer per 100,000 cells (for larger cell samples, i.e.— $1x10^6$ cells, add 500 µL 1X Assay Buffer to cell sample).
7. 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.
8. Spin down cells at 300 x g for 5 minutes in a tabletop centrifuge and discard supernatant.
9. 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.—one million cells, add one mL ice-cold Permeabilization Buffer).
10. Spin down cells at 300 x g for 5 minutes in a tabletop centrifuge and discard supernatant.
11. 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 Not Supplied section on page 3).

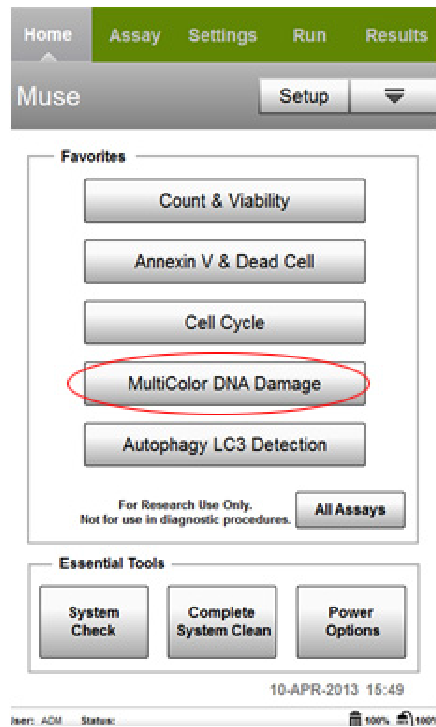
III. Cell Staining and Analysis

12. For multiplexing, add 10 µL of the antibody working cocktail solution as previously described into each microcentrifuge tube containing the cell suspension.
13. Incubate cell testing samples for 30 minutes in the dark at room temperature.
14. Following incubation step, add 100 µL of 1x Assay Buffer to each microcentrifuge testing sample and centrifuge at 300 x g for 5 minutes on a tabletop centrifuge. Discard supernatant.
15. Resuspend cells in each microcentrifuge tube with 200 µL of 1x Assay Buffer.
16. Acquire samples on the Guava Muse Cell Analyzer using the onscreen instructions.

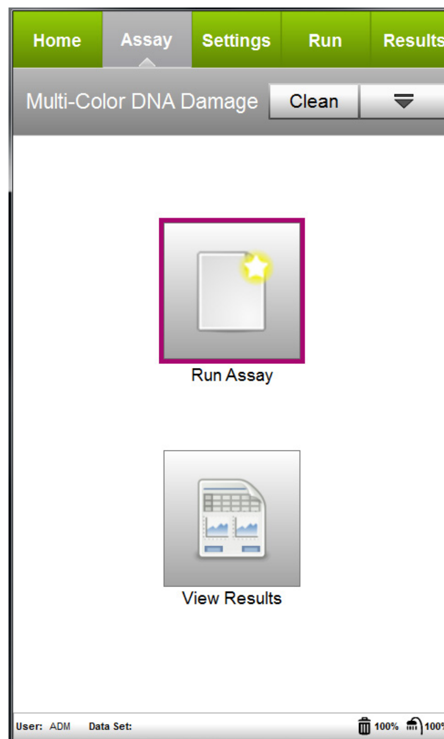
Setup and Acquisition on the Guava® Muse® Cell Analyzer

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.

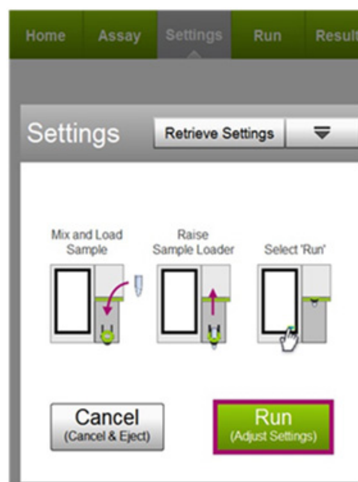
1. Select **MultiColor DNA Damage** from the main menu.



2. Select **Run Assay**.



3. Adjust the instrument settings:



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

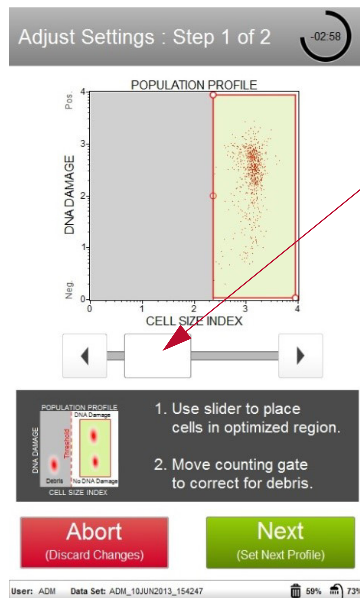
  NOTE: Perform the adjust settings step using a negative control (e.g. no treatment), then verify the settings using a positive control (e.g. treated).

- 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.

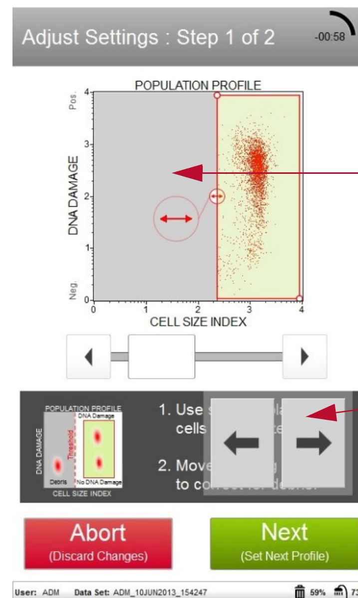
4. Fine tune the settings for the DNA DAMAGE and CELL SIZE INDEX plot, if necessary.
   - Adjust the CELL SIZE INDEX slider accordingly to capture the cell population of interest (see on screen instruction for example).

- Drag the threshold left or right to exclude cell debris. 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 function.

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



Capture cell population of interest by adjusting the CELL SIZE INDEX slider bar on the X-axis.



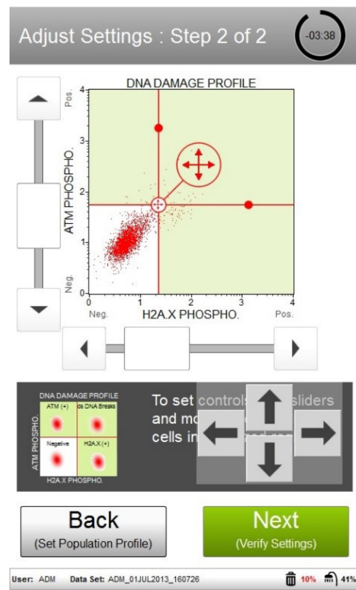
Touch threshold to activate and adjust left/right to exclude cell debris.

Fine tune threshold adjustments by using the arrows below.

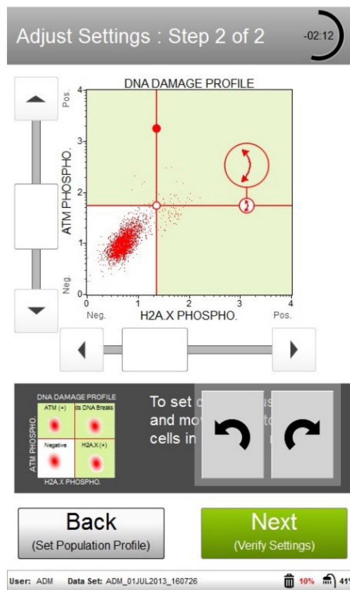
5. Select **Next** when you have completed the adjustments.
6. Fine tune the settings for the ATM (Ser1981) vs. H2A.X (Ser139) plot, if necessary.
   - SETTING THE GATE: To set the quadrant marker properly, prepare a control cell sample (no DNA damage) and place the cell population in the lower left (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), pATM, single positive (UL), pH2A.X, single positive (LR), and DNA double-strand breaks (UR) on scale. If the cell sample is not treated and healthy (e.g. No DNA damage) 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).

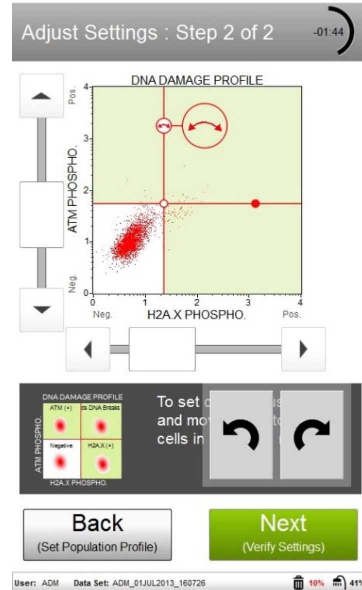
A. Moving the quadrant marker



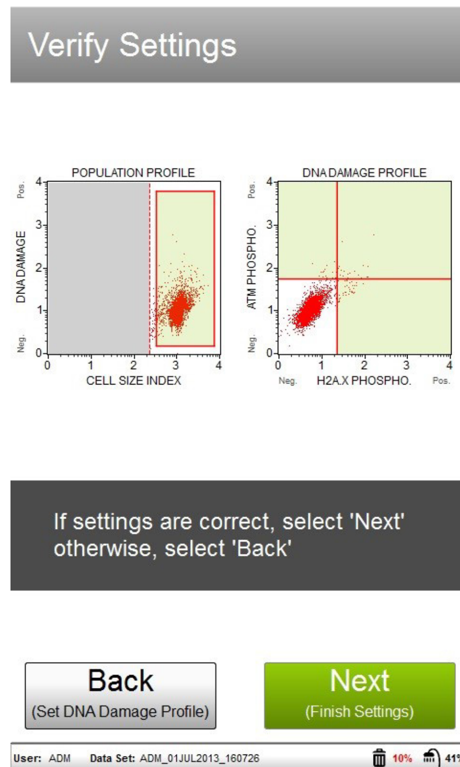
B. Adjusting the X-axis



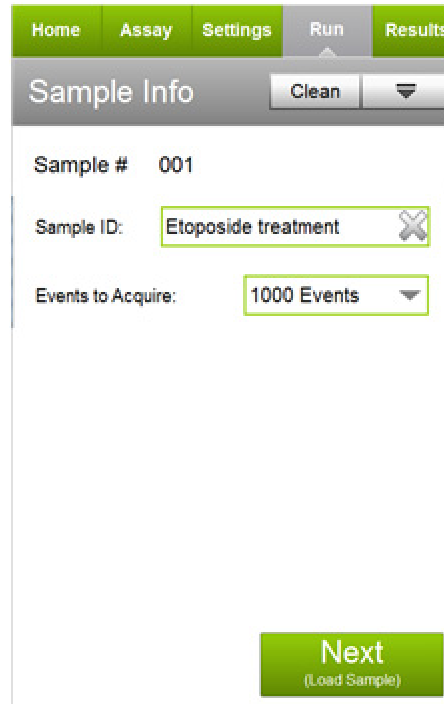
C. Adjusting the Y-axis



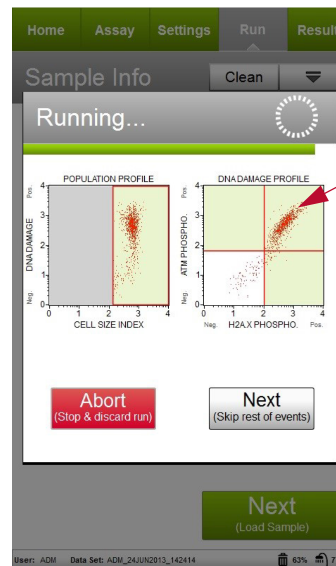
7. Select **Next** when the marker adjustments are complete.
8. Verify the settings. If the settings are correct, select **Next**. Otherwise, select **Back** and repeat steps 4 through 7, as necessary.



9. Enter the sample ID by touching the field, then using the keypad to input the ID. Touch **Done** when you're finished 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**.

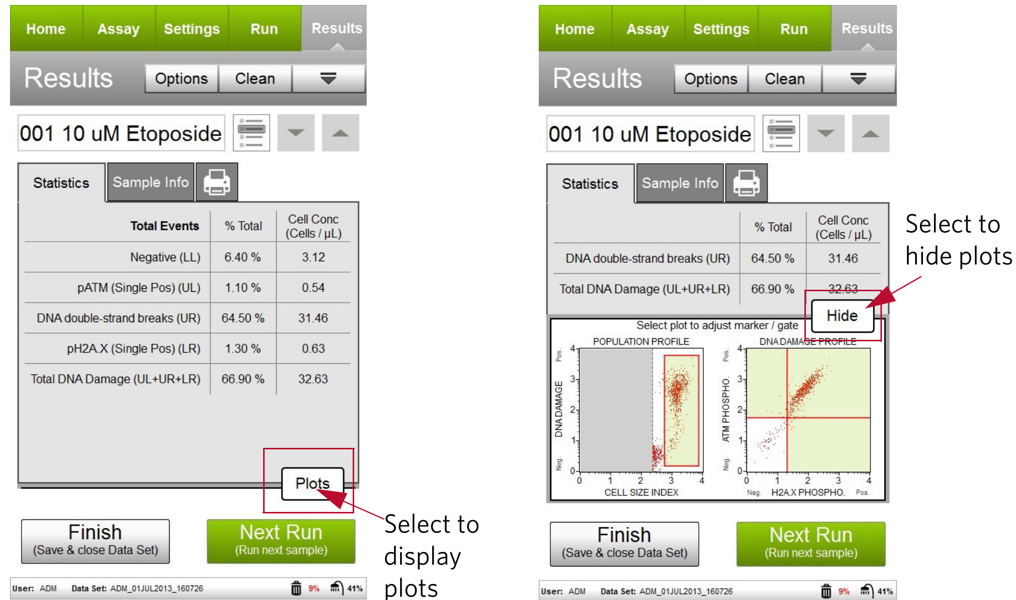


10. Follow the onscreen instructions and mix the first sample. Load the sample on the instrument loading arm. Select **Run** to acquire the sample.



During acquisition, live statistical values are generated and represented on a dot plot graph. Quantitative measurements include Negative, single positive ATM phosphorylation, single positive H2A.X phosphorylation, and DNA double-stranded breaks

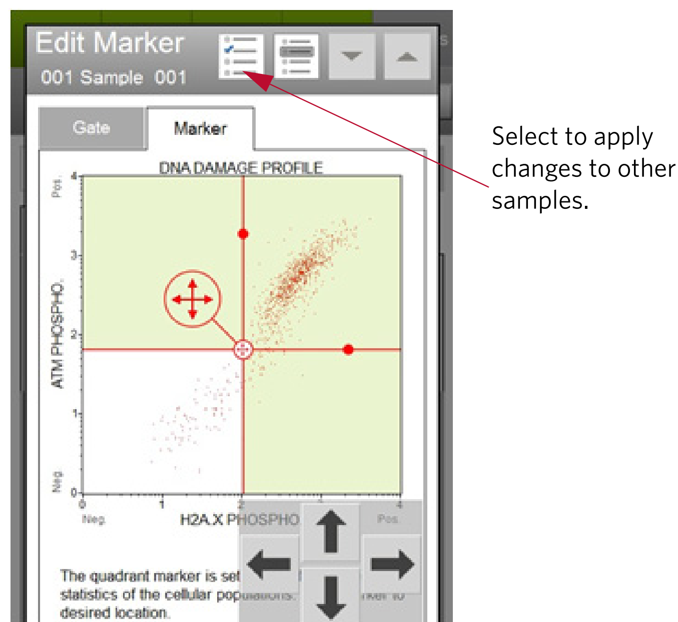
11. When acquisition is complete, the results are displayed. If desired, select **Plots** to display a dot plot and a bar graph for the sample.



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.

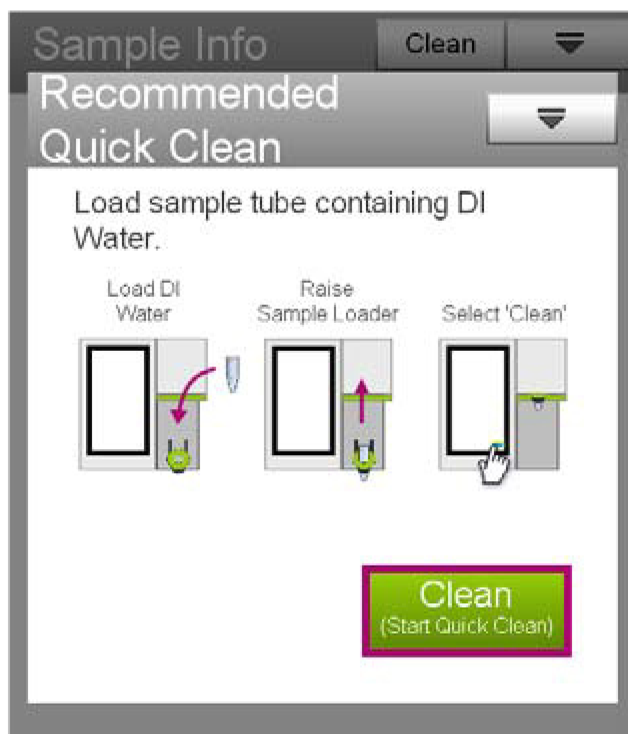
12. (Optional) If changes are needed to the gates assigned, touch the dot plot to enlarge it, then adjust the cell size gate according, as described in steps 4 and 6, respectively. You cannot adjust the cell size threshold after the sample has been acquired.

    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.



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 three ways:

- A dot plot displaying cells which are negative (e.g. no DNA damage), ATM phosphorylated only, H2A.X phosphorylated only, and DNA double-strand breaks (ATM + H2A.X co-activated) on quadrant plot.
- Quantitative percentage (%) measurements of the total events in a table format: Percentages of negative, ATM phosphorylated only, H2A.X phosphorylated only, and dual activation (DNA double strand breaks).
- Summarized DNA damage results: Total DNA damage and DNA double-strand breaks (number of cells per microliter), and total percentage of DNA damage (DSBs).

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:

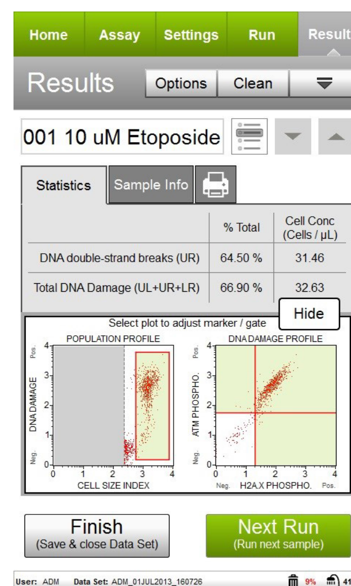
- Sample Number

- Sample ID
- Percent totals for the Negative / only ATM phosphorylated / only H2A.X phosphorylated / Dual activation indicating DNA double-strand break cell types

Statistics | Sample Info

Total Events	% Total	Cell Conc (Cells / µL)
Negative (LL)	6.40 %	3.12
pATM (Single Pos) (UL)	1.10 %	0.54
DNA double-strand breaks (UR)	64.50 %	31.46
pH2A.X (Single Pos) (LR)	1.30 %	0.63
Total DNA Damage (UL+UR+LR)	66.90 %	32.63

Plots



Figures A and B. HeLa cells were exposed to 10 µM Etoposide for 24 hours to induce DNA damage, and then stained with both anti-phospho-Histone H2A.X (Ser139) and anti-phospho-ATM (Ser1981) antibodies in multiplex. Samples were acquired using the Guava® Muse® Cell Analyzer and statistical results are shown above. Figure A shows the results summary, while Figure B shows results displayed in dot plot format.
The statistics captured in this assay show the relative percentages for each population as it is calculated within the total cell population. Cells which express ATM, H2A.X, or both can be seen by the data on upper left, lower right, and upper right quadrants of the dot plot, respectively. In this cell population, 64.5% shows co-activation of ATM and H2A.X upon treatment, indicating DNA damage and double-strand breaks is present.

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 100µL, 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 Multi-Color DNA Damage 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 Multi-Color DNA Damage 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.

For more information, contact Luminex Technical Support.

Troubleshooting

Potential Problems	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. It can be performed at anytime 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.
High Cell Concentration warning during acquisition	If the concentration of the stained cell sample is high (>1200 cells/µL), dilute the sample further with Assay Buffer 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.

Related Products

1. Muse® H2A.X Activation Dual Detection Kit (Catalog No. MCH200101)
2. Muse® EGFR-RTK Activation Dual Detection Kit (Catalog No. MCH200102)
3. Muse® PI3K Activation Dual Detection Kit (Catalog No. MCH200103)
4. Muse® MAPK Activation Dual Detection Kit (Catalog No. MCH200104)

5. Muse® Bcl-2 Activation Dual Detection Kit (Catalog No. MCH200105)
6. Muse® PI3K/MAPK Dual Pathway Activation Kit (Catalog No. MCH200108)
7. Muse® System Check Kit (Catalog No. MCH100101)
8. Muse® Count & Viability Kit (100T) (Catalog No. MCH100102)
9. Muse® Annexin V & Dead Cell Kit (Catalog No. MCH100105)
10. Muse® Cell Dispersal Reagent (Catalog No. MCH100107)

References

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