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Guava® DNA Damage Histone H2A.X Dual Detection Kit User's Guide

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Application

The Guava® Dual Detection kits are a series of flow cytometry products which include a pair of antibodies that bind to the same protein; one to detect total protein expression and another to detect the phosphorylated form of the same target. Using two parameter analysis, we can achieve target specific detection of phosphorylation and, by doing so, eliminate false positives while enhancing the signal to noise ratio.

H2A.X is a member of the histone H2A family. Histone H2A.X resides downstream of the DNA damage kinase signaling cascade. Phosphorylation of Histone H2A.X at serine 139 is an important indicator of DNA damage.[1] As the level of DNA damage increases, the level of phospho Histone H2A.X (also known as γH2AX) increases, accumulating at the sites of DNA damage. This accumulation of phospho Histone H2A.X is often used to indicate the level of DNA damage present within the cell.[1] H2AX is also responsible for recruiting response proteins to the site of DNA damage and may play a role in DNA repair.[2]

In order to validate that the level of H2A.X activation is accurate, a total H2A.X antibody is multiplexed by flow cytometry to allow the total and phospho levels to be normalized since DNA content levels vary based on which stage cells are within the cell cycle. Histone doubles in content during the cell cycle at the same rate DNA content doubles.[1] Having a total H2A.X will account for this increase, so the phosphorylated levels can be accurately determined.

In all, a comprehensive understanding of Histone H2A.X activity and understanding the consequence of its activation can provide the researcher with useful information which will be important in understanding the intrinsic nature of the DNA damage response. The Guava Histone H2A.X DNA Damage Dual Detection Kit is designed to allow the researcher to monitor and accurately measure phospho-specific Histone H2A.X activation in a population of cells.

All Guava kits are optimized on Guava® bench top flow cytometers. Guava kits can be used on any flow cytometer following the same protocol providing researchers a reliable and fully validated solution to study the Histone H2A.X signaling pathway right in the comfort of their own lab. 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, wash, and assay buffers to provide researchers with a complete solution for H2A.X signaling analysis.

Test Principle

The Guava® Histone H2A.X DNA Damage Dual Detection kit includes two directly conjugated antibodies, a phospho-specific Anti-phospho-Histone H2A.X (Ser139)-PerCP and an Anti-Histone H2A.X-FITC conjugated antibody to measure total levels of Histone H2A.X. This two color kit is designed to detect the extent of Histone H2A.X pathway activation by measuring H2A.X phosphorylation relative to the total H2A.X expression in any given cell population. By doing such, the levels of both the total and phosphorylated protein can be measured simultaneously in the same cell, resulting in a normalized and accurate measurement of H2A.X activation after stimulation. Moreover, simultaneous measurement of both total and phospho-Histone H2A.X confirms target specificity of the phosphorylation event. Together, a total and phospho antibody duo performed in multiplex provides an enhanced and more reliable detection of the phospho: total ratio within a mixed cell population. Using this antibody pair provides a sensitive and valuable tool to study the factors that induce DNA damage and/or affect DNA repair, and allow 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 level 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.[3] H2A.X phosphorylates in response to a DNA damaging reagent (e.g., Etoposide) or UV light, and its activation clearly indicates that DSBs have occurred.

Understanding when DSBs take place can help researchers understand the mechanisms involved in DNA repair and the DNA damage response.

The antibody pair provided in the kit have been carefully titrated to ensure the ability to measure Total and phospho-Histone H2A.X simultaneously on the same protein for accurate determination of protein level and activation. Sufficient reagents are provided to perform 25 two-color tests. Detailed assay instructions are included to assist in analysis and to ensure the correct cell concentration is obtained during acquisition of sample data.

Case Study: Assessment of DNA damage using topoisomerase inhibitor, Etoposide (and various other small molecules)

A case study was conducted to evaluate the effects of etoposide treatment on HeLa cells. Proliferating cells are especially vulnerable to DNA damage due to the added demands of cellular growth and division. DNA topoisomerase inhibitors induce lethal chromosome damage, including breaks and rearrangements.[4] When stimulating HeLa cells with etoposide for 2 hours a marked increase in phosphorylated H2A.X is detected (figure 3). In our assay design using flow cytometry, the total H2A.X protein level remains constant compared to unstimulated cells, while phospho- Histone H2A.X levels are increased in all cells indicating that there is no competition between the two antibodies for their target epitopes. The absence of interaction makes this dual detection method attractive and sensitive when evaluating DNA stranded breaks.

To further investigate the effect of anti-neoplastic agents on DNA damage, deep dive analysis was conducted by titration of various small molecules to define EC50 values (figures 4 and 5). Since structure-activity relationships (SAR) of small molecules are critical in identifying selective anti-neoplastic agents, the degree of H2A.X phosphorylation was determined by flow cytometry as indicated by the mean fluorescence intensity of the signal. In figure 4, two critical time points were determined for etoposide analysis, 2-hour incubation and 24-hour incubation. From these values, a dose response curve is developed and EC50 values determined. In figure 5, other various small molecules are evaluated after 24 hour incubation on HeLa cells and compound comparisons are then determined. By implementing this method anti-tumor compounds can be rank ordered to help complement any SAR campaigns during drug development.

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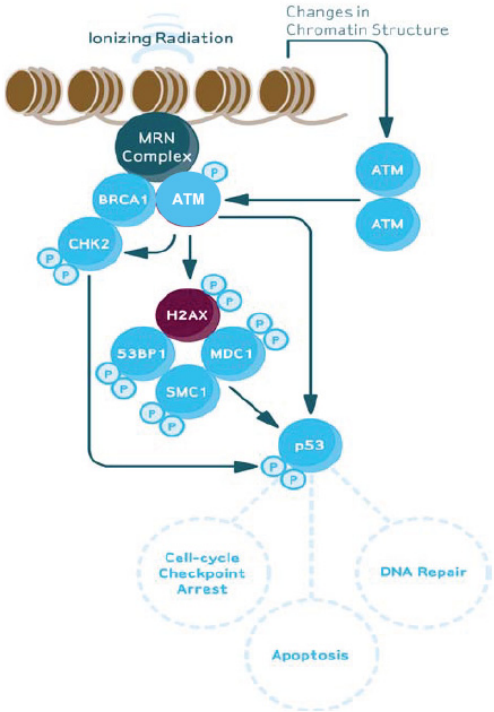


The DNA Damage Signaling Pathway

Kit Components

- 20X Anti-phospho-Histone H2A.X (Ser139)-PerCP (Part No. CS206553), One vial containing 150 µL
- 20X Anti-Histone H2A.X-FITC (Part No. CS206429), One vial containing 150 µL
- Fixation Buffer (Part No. CS202122), One bottle containing 13 mL
- 10X Wash Buffer (Part No. CS202123), One bottle containing 13 mL
- 5X Assay Buffer (Part No. CS202124), One bottle containing 55 mL
- 1X Permeabilization Buffer (Part No. CS203284), Two bottles containing 14 mL

Materials Not Supplied

- 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.
- Pipettors with corresponding tips capable of accurately measuring 10–1000 µL
- Tabletop centrifuge capable of achieving 300 x g
- Mechanical vortex
- Flow Cytometer
- Deionized water (for Buffer dilutions)
- HeLa cells or cells of interest
- Etoposide reagent (EMD Chemicals; Part No. 341205)
- Isotype control; rabbit IgG- FITC (based on user preference)
- Guava® ViaCount™ Reagent (Part No. 4000-0040)

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. Kit contains a fixation solution containing paraformaldehyde. Refer to the Safety Data Sheet (SDS) for specific information on hazardous materials (SDS forms can be found by contacting Luminex technical services).
- The conjugated antibody is light sensitive and must be stored in the dark at 2-8 °C.
- During storage and shipment, the product supplied in vials may condense within the vial. For maximum recovery of the product, centrifuge original vial prior to removing cap.
- Do not use reagents beyond the expiration date of the kit.

Storage

This kit must be stored at 2–8°C. The 10X Wash Buffer (Part No. CS202123) and Fixation Buffer (Part No. CS202122) can be stored at either 2–8°C or at room temperature upon receipt.
Caution: The fluorochrome-conjugated antibody should always be stored at 2–8°C and stored in the dark.

Please refer to your product label for information on the product lot, including the expiration date. Details on the Luminex Reagent Shipping Policy, including any shelf-life guarantees, can be located at luminexcorp.com. **Please avoid repeated changes in temperature as this will affect the integrity of the product.**

Preparation of Reagents

Wash Buffer

Wash Buffer is supplied at 10X concentration and should be diluted to 1X with deionized water prior to use. Prepared 1X Wash Buffer is stable up to one year. Store at 2–8°C.

NOTE: 10X Wash Buffer can be stored at RT to avoid any potential precipitate formation

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.

Assay Instructions

NOTE: This assay protocol has been optimized for human HeLa cells. However, this kit is suitable for measuring the extent of 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.

Flow Kit Staining Protocol

NOTE: During all steps in the assay procedure, keep all reagents on ice. After preparation of the cell cultures, follow the guidelines listed to ensure proper cell staining for optimal analysis.

I. Cell Culture and Stimulation

1. Seed 10 million HeLa cells into two T-75 flasks (5 x 10^6 cells / flask) overnight in a 37°C incubator with 5% CO_2. Cells should be at about 90% confluent the next day.
2. Add 20 mL of media to one flask and label it *Untreated*.
3. Add 20 mL of media to the other flask and label it *Treated*.
4. The next day, replace the media in both flasks with 20 mL of fresh media.

5. To the Treated flask add 20 µL of 100 mM Etoposide (in DMSO) for a final concentration of 100 µM. To the Untreated flask add 20 µL of DMSO.
6. Incubate the flasks in a 37°C incubator with 5% CO_2 for 2 hours.
7. After 2 hours incubation, aspirate the media and rinse both T-75 flasks with 5 mL HBSS.
8. Add 1 mL of accutase in DPBS, 0.5-mM EDTA (Millipore # SCR005) to each flask and incubate at 37°C for 5 minutes; if necessary, tap gently to dislodge the cells.
9. Deactivate accutase with 9 mL of media and put the cells from the Treated flask into a 15-mL conical tube labeled *Treated* and put the cells from the untreated flask into a 15-mL conical tube labeled *Untreated*.
10. Determine cell numbers by using ViaCount™ or a hemacytometer and note cell viability. Healthy cells should be above 90% viable.

II. Fix and Permeabilize Cells

1. After deactivating accutase count the cells, spin down the Treated and Untreated conical tubes at 300 x g for 3 minutes and discard the media.
2. Resuspend cells by adding 500 µL of 1X Wash Buffer per 500,000 cells (for larger cell samples, i.e.,— 1 x 10^6 cells, add 1 mL 1X Wash Buffer to cell sample). Essentially, add 100 µL of 1X Wash Buffer for every extra 100,000 cells evaluated.
3. Add equal parts Fixation Buffer to 1X Wash Buffer (1:1). So for every 500 µL of 1X Fixation Buffer per 500,000 cells, add an additional 500 µL Fixation Buffer for a total of 1 mL cell fixation solution, and mix sample by pipetting up and down. (Similarly, add 100 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 x g for 3 minutes in a 4°C centrifuge and discard buffer.
5. Resuspend cells in 1 mL of 1X Assay Buffer per 1 x 10^6 cells.
6. Spin down cells at 300 x g for 3 minutes in a 4°C centrifuge and discard buffer.
7. Permeabilize cells by adding 1 mL ice-cold 1X Permeabilization Buffer per 1 x 10^6 cells and incubate on ice for 20 minutes (For smaller cell samples, i.e.,—500,000 cells, add 500 µL ice-cold 1X Permeabilization Buffer).
8. Spin down cells at 300 x g for 3 minutes in a 4°C centrifuge and discard buffer.
9. Resuspend cells in 0.5 mL 1X Assay Buffer per 1 x 10^6 cells and add 250 µL of cells into a V-bottom 96-well plate. (See manual for instrument compatible plates). *Alternatively, centrifuge tubes can be used for sample staining and further preparation.
10. Spin down cells at 300 x g for 3 minutes in a 4°C centrifuge and discard buffer.
11. Wash cells with 200 µL of 1X Assay Buffer per well,then repeat.

III. Cell Staining and Flow Analysis

1. For single-color staining, resuspend the cells in 95 µL of assay buffer and add 5 µL of either Anti-phospho-Histone H2A.X-PerCP or Anti-Histone H2A.X-FITC to each sample well.
2. For multiplexing, resuspend the cells in 90 µL of assay buffer and add 5 µL of Anti-phospho-Histone H2A.X-PerCP and add 5 µL of Anti-Histone H2A.X-FITC to each sample well.
3. Incubate cells for 30 minutes in the dark at room temperature.
4. Add 100 µL of 1X Assay Buffer to the 100 µL of diluted antibodies already in the wells and centrifuge at 300 x g for 5 minutes at 4°C. Discard supernatant.

5. Wash with 200 µL of 1X Assay Buffer and centrifuge cells at 300 x g for 5 minutes at 4°C. Discard supernatant.
6. Resuspend cells in each well with 200 µL of 1X Assay Buffer.
7. Perform flow cytometry analysis.

Sample Data

Figure 1: Analyzed Single-Parameter Data for Anti-phospho-Histone H2A.X (Ser139) – HeLa cells were treated with 100 µM of etoposide and then stained with phospho-Histone H2A.X – PerCP (red). Untreated HeLa cells (grey) were also stained and results are shown overlaid in each plot.

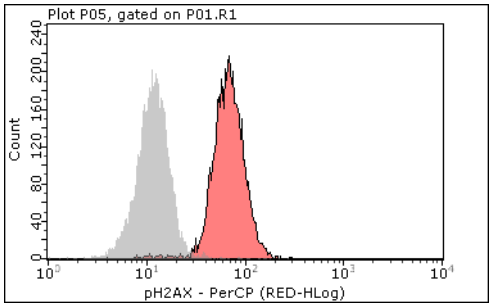
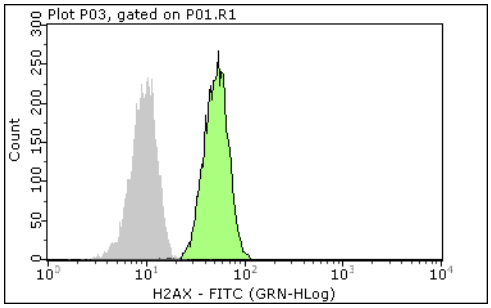


Figure 2: Analyzed Single-Parameter Data for Anti-Histone H2A.X (total H2A.X) on HeLa cells – Untreated HeLa cells were stained with Anti-Histone H2A.X- FITC (green) versus and isotype control (grey).



Two parameter analysis using total phospho antibodies

Figure 3: Dual Parameter Analysis of Total and Phospho Histone H2A.X on HeLa Cells Unstimulated HeLa cells are stained with both phospho-Histone H2A.X-PerCP and Anti-Histone H2A.X-FITC (A), where there is no indication of Histone H2A.X activation via phosphorylation, but only on total H2A.X as noted by 97.2% of cells. However, once HeLa cells were stimulated with 100 µM etoposide, simultaneous measurement of both total and phospho

Histone H2A.X confirms target specificity of the phosphorylation event as indicated by the double positive cell population (B) as indicated by the 2.09% to 97.15% increase of double positive staining.

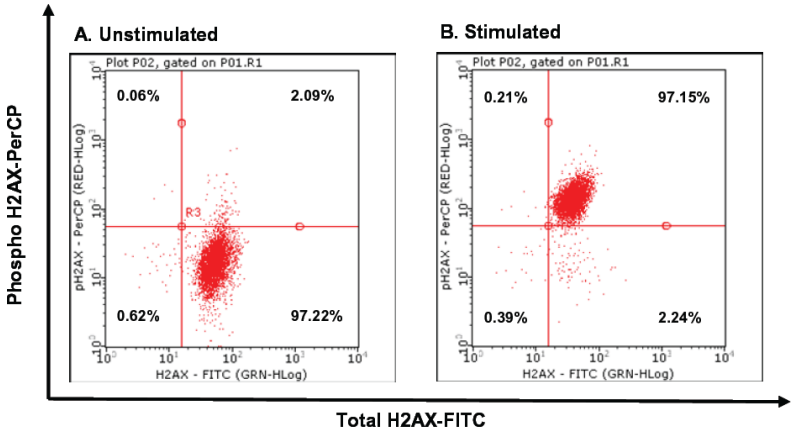


Figure 4: Dose response curves for topoisomerase inhibitor, Etoposide HeLa cells were treated with etoposide for either 2 hours (A), or for 24 hours (B). As indicated by the dose response curves, it has been demonstrated that using a phospho-specific antibody by flow cytometry can function as a legitimate screening tool to evaluate anti-neoplastic agents (change in mean fluorescence values provide the data points). Moreover, kinetic studies by longer incubation times indicate that compound efficacy can be influenced greatly, providing the researcher with valuable information when identifying advance leads.

A. 2-hour incubation

B. 24-hour incubation

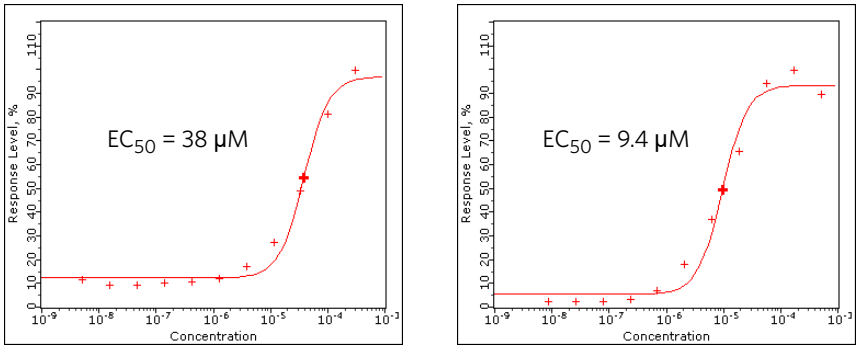
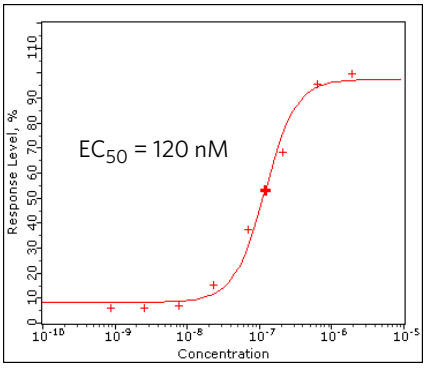


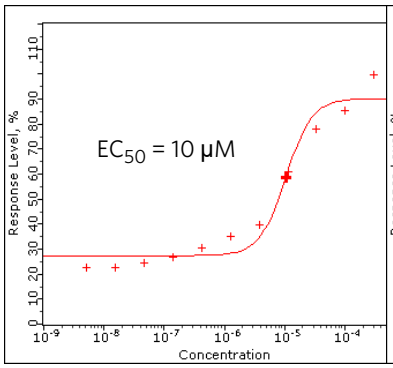
Figure 5: Compound rank ordering by flow cytometry – HeLa cells were pre-treated with various small molecule inhibitors for 24 hours prior to staining and acquisition. EC50 curves are derived from half log serial dilutions and

samples are then rank ordered based on their efficacy by using a phospho-specific H2A.X–PerCP antibody. Compound Rank Order: Topotecan > Etoposide ≥ Anisomycin > Aphidicolin

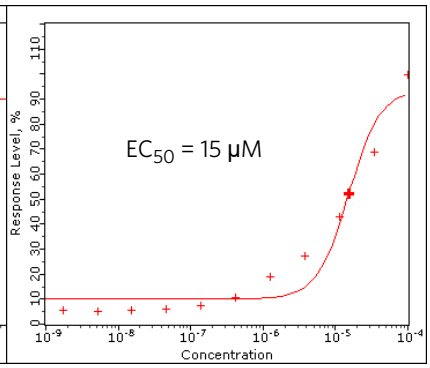
A. Topotecan



B. Anisomycin



C. Aphidicolin



Technical Hints

- If minor precipitate is detected in the 10X Wash Buffer place the bottle in a warm water bath for 30 minutes, followed by mixing the contents on a mechanical vortex.
- For cellular staining and analysis to be most effective, make sure that test cells have good viability prior to use.
- 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).
- Do not mix or interchange reagents from various kit lots.

Troubleshooting

Assay Step	Potential Problem	Experimental Suggestions
Reagent Preparation	Precipitation found in 10X Wash Buffer	If storing at –20°C, precipitate can form in the 10X wash buffer. Prior to use, place bottle in a 37°C water bath, swirling the contents occasionally. If this does not remove the precipitate completely, allow 10X Wash Buffer to sit at room temperature overnight.

Assay Step	Potential Problem	Experimental Suggestions
Acquisition	Acquisition rate decreases dramatically	This usually indicates that the fluid pathway on the instrument may be blocked. This can be alleviated by the following: • Decreasing number of cells for analysis. Guava® flow cytometers have the capacity of analyzing a steady stream of 300–500 cells per microliter. Any cell densities in excess can essentially block the normal flow, causing disruption during the assay. Decrease the number of cells being analyzed by diluting the sample to approximately 0.5 million cells per milliliter. • Adherent or sticky cells can result in cellular clumping. Use a more aggressive enzyme for dissociation such as trypsin during cell harvesting should help keep cells in single suspension. Alternatively, using a cell strainer can help disrupt cell clumping if needed (Catalog No. SCNY00060; 60 µM) • After many uses, it is possible that the fluid system on any standard flow cytometer will require cleaning. Run standard cleaning procedures to clean the fluid system during or after an assay. This will prevent any material from forming where the steady flow steam takes place.
Cellular Analysis	A loss or lack of signal	• Cell numbers may need to increase. Cell loss is common during washing steps in the assay procedure. A substantial decrease in cell numbers can lead to a loss of signal. Make sure that cell density remains at approximately 0.5 million cells per milliliter during analysis. • Although the assay procedure has been optimized to function utilizing many different cell types, further antibody titrations may be necessary for some cell types to capture the ideal staining concentration. A lack of signal may indicate that excess antibody will need to be used during the staining procedure.
	Background and/or non-specific staining of cells	Although the assay procedure has been optimized to function utilizing many different cell types, further antibody titrations may be necessary for some cell types to capture the ideal staining concentration. Non-specific staining and background may indicate that less antibody will need to be used during the staining procedure.
	Variability in day-to-day experiments	• 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. • When using the Guava easyCyte™ Plus instrument for flow analysis, make sure that a quality check on the instrument (e.g., calibration) is performed on a daily basis prior to use. (*See Analytical Sensitivity and Detection Limits Section for Guava Check standards)

References

1. Tanaka T, et al. Cytometry of ATM Activation and Histone H2AX Phosphorylation to Estimate Extent of DNA Damage Induced by Exogenous Agents. *Cytometry*. 2007;71A:648-661.
2. Ewald B, et al. H2AX phosphorylation marks gemcitabine-induced stalled replication forks and their collapse upon S-phase checkpoint. *Mol Cancer Ther*. 2007;6(4):1239–1248.
3. Muslimovic A, et al. (2008). An optimized method for measurement of gamma-H2AX in blood mononuclear and cultured cells. *Nat Protoc*. 2008;3(7):1187-1193.
4. Kaufmann WK, et al. DNA damage and cell cycle checkpoints. *The FASEB Journal*. 1996;10:238-247.

Related Products

- Guava® DNA Damage Histone H2A.X Dual Detection Kit ViaCount™ (Cat No. 4000-0040)
- Guava® easyCheck™ (Cat No. 4500-0025)