FlowCellect™ Cell Cycle Checkpoint ATM DNA Damage Kit

25 Tests

Cat. No. FCCH025143

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Not for use in diagnostic procedures

USA & Canada Phone: +1 (800) 437-7500 Fax: +1 (951) 676-9209
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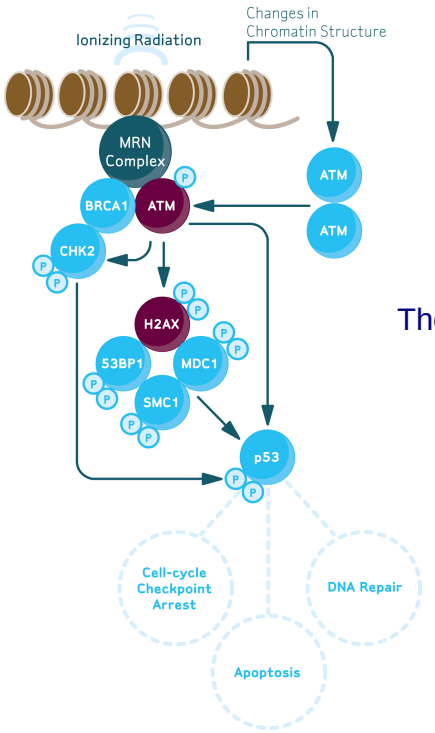
Application

Investigating the DNA damage signaling pathway is an important area for genome health and cancer research. Evidence suggests there is a direct correlation between DNA damage and cell cycle. Cells that are defective in DNA damage 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 Ataxia telangiectasia 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 [6].



The DNA Damage Signaling Pathway

Millipore's FlowCellect™ DNA Damage (ATM) and Cell Cycle Analysis Kit is designed to identify the linkage between DNA damage in relation to cell cycle checkpoints in flow cytometry applications. The kit includes a directly conjugated Anti-phospho-ATM (Ser1981) Alexa Fluor® 488 conjugate plus a DNA dye (Propidium Iodide) which allows the researcher to perform bivariate analysis to identify where DNA damage takes place relative to the positioning within the cell cycle. The kit includes an optimized protocol with all of the necessary components to provide the researcher a true "plug and play" design for their research when studying DNA damage and cell cycle checkpoints. This immunocytochemical method is very sensitive, and additionally it offers the capability to screen anti-tumor drugs at every phase of the cell cycle.

In all, a comprehensive understanding of DNA damage and its association with cell cycle behavior can provide the researcher with useful information which will be important in interpreting the intrinsic nature of cell proliferation, apoptosis, DNA repair, and assist in the development of anti-neoplastic agents. Millipore's FlowCellect™ DNA Damage (ATM) and Cell Cycle Analysis Kit is designed to allow the researcher to easily identify, discriminate, and quantitate cells undergoing DNA damage relative to their positioning within the cell cycle.

All FlowCellect kits are optimized on guava® bench top flow cytometers. FlowCellect kits can be used on any flow cytometer following the same protocol providing researchers a reliable and fully validated solution to study double stranded breaks (DSBs) and cell cycle distribution right in the comfort of their own lab. This kit contains optimized fixation, permeabilization, wash, and flow buffers to provide researchers with a complete solution for bivariate analysis to assess DNA damage in relation to positioning within the cell cycle.

Test Principle

Ataxia telangiectasia mutated (ATM) is a protein kinase that becomes activated in response to DNA damage, particularly when the damage involves formation of DNA double stranded breaks [2]. Detection of DNA damage due to DSBs is a fundamental method for assessing cell health and evaluation of anti-neoplastic agents. Bivariate analysis monitoring the extent of ATM phosphorylation along with a DNA dye offers a sensitive and valuable tool in studying the factors that induce DNA damage (measured by the level of DSBs) and/or affect DNA repair [2]. Understanding where the first indication of DSBs take place during the cell cycle can help researchers understand the mechanisms involved in cell cycle arrest and DNA repair at critical cell cycle checkpoints [2, 3, 5]. Malfunctions in the cell cycle in response to DNA damage can increase the incidence of cancer since the DNA repair machinery fails to operate properly. This is important because the cell has several systems for interrupting the cell cycle if the DNA repair machinery malfunctions. ATM has been shown to colocalize at sites of ionizing radiation induced DNA damage and to phosphorylate the histone variant H2AX on Ser139. Evidence suggests that H2A.X phosphorylation may recruit checkpoint proteins to sites of DNA damage and play a role in DNA repair [4].

Various factors can cause DNA double stranded breaks resulting in DNA damage. Physical factors such as UV and ionizing radiation, or chemical factors such as synthetic compounds (anti-tumor drugs) can all cause DNA damage. Regardless of the source to induce DNA damage, the end result will either cause cell cycle checkpoint arrest, apoptosis, or will trigger the DNA repair response to damage.

A case study was performed to validate the useful application of this kit by accurately measuring and quantitating the extent of DNA damage and how it relates to positioning within the cell cycle. HeLa cells were treated with well known topoisomerase inhibitors, Topotecan and Etoposide. DNA topoisomerases (topo1 and topo2) are among the most effective anti-tumor drugs currently available. Their mode of action is thought to involve stabilization of ‘‘cleavable’’ complexes between topo1 or topo2 and DNA that are transiently formed during DNA replication or untangling (Late G1 → early S-phase inhibitors). Collisions of the progressing DNA replication forks or the progressing RNA polymerase molecule with the stabilized complexes convert them into DSBs, which are recognized as lethal lesions and trigger apoptosis. Predominantly S-phase cells undergo apoptosis after exposure to topo1 inhibitors such as Topotecan [2, 3]. Etoposide, which is a topo2 inhibitor, induced maximal DNA damage (ATM phosphorylation) during G1 and triggered apoptosis in all phases of the cycle [2]. Lastly, it has been reported that exposure to UV light triggers DNA damage response (DDR) as seen by the phosphorylation of ATM on Ser1981, not only in S- but in G1-phase cells [7].

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.

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Kit Components

1. 20X Anti-p-ATM (Ser1981), clone 10H11.E12 - Alexa Fluor® 488 Conjugate: (Part No. CS206400) One vial containing 150 µL antibody.
2. Propidium Iodide Solution: (Part No. CS204836) One vial containing 400 µL DNA dye.
3. RNase A Reagent: (Part No. CS204821) One vial containing 25 µL.
4. Fixation Buffer: (Part No. CS202122) One bottle containing 13 mL buffer.
5. 10X Wash Buffer: (Part No. CS202123) One bottle containing 13 mL buffer.
6. 5X Assay Buffer: (Part No. CS202124) One bottle containing 55 mL buffer.
7. Permeabilization Buffer: (Part No. CS203284) Two bottles containing 14 mL buffer.

Materials Not Supplied

1. Test tubes for sample preparation and storage
2. Tissue culture reagents, i.e. HBSS, PBS w/o Ca^{2+} or Mg^{2+}, cell dislodging buffers, etc.
3. Pipettors with corresponding tips capable of accurately measuring 10 – 1000 µL
4. Tabletop centrifuge capable of exceeding 400 x g
5. Mechanical vortex
6. Flow Cytometer
7. Deionized water (for Buffer dilutions)
8. Dimethyl sulfoxide (DMSO)

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. Please refer to the MSDS sheet for specific information on hazardous materials (MSDS forms can be found by contacting Millipore technical services).
- The conjugated antibody is light sensitive and must be stored in the dark at 4°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 - 8ºC. The 10X Wash Buffer (Part # CS202123) and Fixation Buffer (Part # CS202122) can be stored at either 2 - 8ºC or at room temperature upon receipt. **Caution:** The fluorochrome conjugated antibody should always be stored at 2 - 8ºC and stored in the dark. Propidium Iodide Solution can be stored at -20℃ fo r long term storage, but avoid freeze/thaw.

All kit components are stable up to six (6) 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.**

Preparation of Reagents

1. **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 - 8ºC.

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

2. **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 - 8ºC.

3. **Propidium Iodide/RNase solution**

   Propidium Iodide Solution is provided in the kit at a concentrated form. For every experiment, dilute Propidium Iodide Solution 1:12 in 1X Assay Buffer (one part PI solution to 11 parts 1X Assay Buffer). Following dilution of PI, directly add RNase solution at a 1:300 dilution (e.g. for 1 mL of diluted PI solution, add 3.3 µL RNase). Prepare fresh solution for each assay run. Keep fresh solution at room temperature prior to use.

Assay Instructions

Note: This assay protocol has been optimized for human HeLa cells. However, this kit is suitable for measuring the extent of DNA damage and cell cycle analysis of 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.

1. Harvest Cells

For adherent cells, treat cell culture by using a mild enzyme such as trypsin or an EDTA-based cell dissociation buffer.

For suspension cells, treat cell culture by gently pipetting cell suspension up and down to ensure complete homogeneity. Remove cell culture media by centrifugation at 250 x g for 5 minutes.

*NOTE: It is recommended to prepare two extra samples for single color staining; one sample for phospho-ATM staining only (using pATM positive cells) and one sample for PI staining only. These samples will be used for compensation of test samples. Please refer to the compensation guide below for more details.

2. Fix and Permeabilize Cells

- For every $1x10^6$ cells, resuspend with 100 µL of 1X Wash Buffer and add 100 µL of Fixation Buffer. Incubate cells for 20 minutes on ice.
- Remove fixation buffer by centrifugation at 670 x g for 5 minutes. Aspirate and discard supernatant, only saving the cell pellet.
- For every $1x10^6$ cells, resuspend with 100 µL ice cold 1X Permeabilization Buffer. Allow to incubate for 20 minutes on ice.
- Remove Permeabilization Buffer by centrifugation at 670 x g for 5 minutes. Aspirate and discard supernatant, only saving the cell pellet.
- Wash cells once by adding 200 µL 1X Assay Buffer for every $1x10^6$ cells. Carefully disrupt cell pellet to homogeneity, and remove assay buffer by centrifugation at 670 x g for 5 minutes. Aspirate and discard supernatant, only saving the cell pellet.

3. Antibody Staining

- Add 5 µL of 20X stock Anti-phospho-ATM (Ser1981) direct conjugate to Alexa Fluor 488 to 95 µL of 1X Assay Buffer for every $1x10^6$ cells stained. Allow to incubate on ice for 30 minutes in the dark.
- Wash cells once by adding 200 µL 1X Assay Buffer for every $1x10^6$ cells. Again, carefully disrupt cell pellet to homogeneity, and remove assay buffer by centrifugation at 670 x g for 5 minutes. Aspirate and discard supernatant, only saving the cell pellet.

4. DNA Staining

Add 200 µL of freshly prepared Propidium Iodide/RNase solution (cells in a final concentration of $0.5x10^6$ cells per mL). Incubate for 30 minutes at room temperature in the dark.

5. Analysis

- All DNA measurements (PI staining plotted on the X-Axis) MUST be analyzed using linear scaling. Analysis using logarithmic scale will make it difficult to discern cellular distributions for each phase of the cell cycle.
- Voltage adjustment: In order to differentiate G1, S and G2 phase cell populations, increase the voltage accordingly until a good degree of cell separation is achieved
- Performing cell analysis on a guava easyCyte 8HT instrument can be performed by using 96-well plate (200 µL volumes) or single tube (500 µL volumes).
- If using alternative flow cytometry instrumentation, dilute cells according to instrument manufacturer's recommendations.

Doublet Discrimination (Area vs. Width)

During cell cycle analysis, when using a DNA dye such a propidium iodide it is of critical importance to eliminate cell aggregates. Cell aggregates contain a greater number of DNA since each aggregate account for two or more cells clumped together. This can cause misleading results, especially when differentiating cells in G1 phase (2C) or cells in G2 phase (4C). Without addressing doublet discrimination, the question arises when measuring DNA Content: are we measuring 4C cells or 2C doublets?

Doublet discrimination can be achieved by implementing gating strategies plotting area versus width. By doing such, it can be easily determined which cells are truly in single cell suspension versus cells which are clumped together based on the increased width of a given cell population.

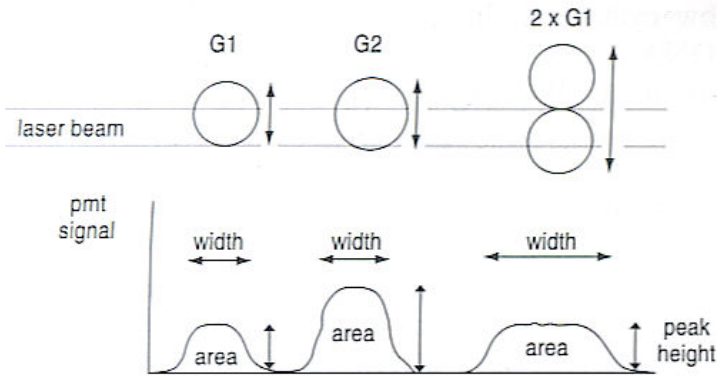

Figure 2. Time of cell flight distinguishes doublets from single cells in suspension As cells pass through the point of interrogation, the laser beam width and area increases as the width of the cell increases. Hence, a larger cell (e.g. aggregates) will cause an increase in cell width.

A. Ungated sample **B. Gated sample**

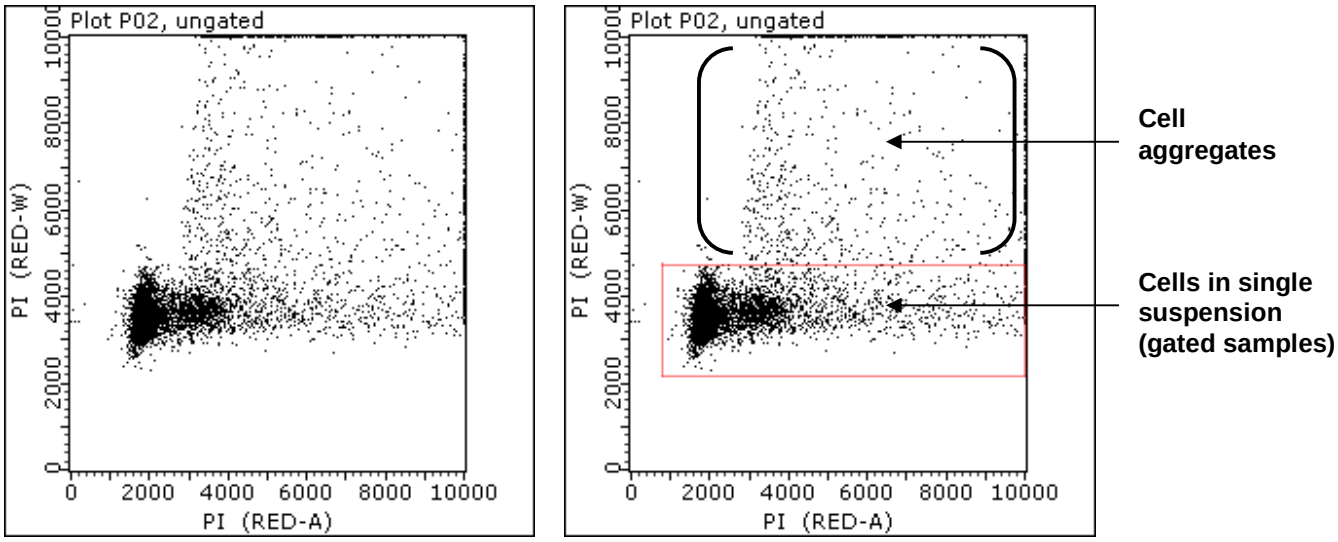

Figure 3. Doublet discrimination: Plotting Area vs. Width Aggregate G1 cells can mistakenly be interpreted as G2 cells, leading to inaccurate results. Eliminate false G2 cells by gating only accounting for cells giving a constant width. Plot A shows an ungated sample containing excessive cell scattering which will account for all the cell aggregates or coincidence events. In B, only cells of interest are used for analysis purposes.

Sample Results

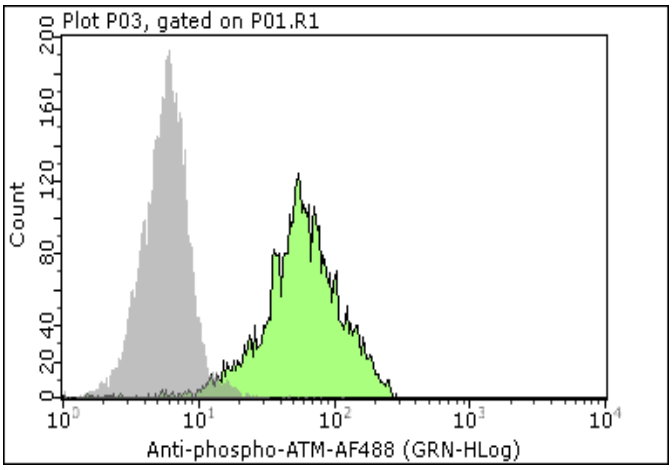

Figure 3. Anti-phospho-ATM (Ser1981) antibody validation using Etoposide Flow cytometery analysis of etoposide treated versus untreated HeLa cells stained with Anti-phospho-ATM (Ser1981) Alexa Fluor 488 antibody. Cells treated with 100 µM etoposide are shaded in green, compared to the untreated sample shaded in grey.

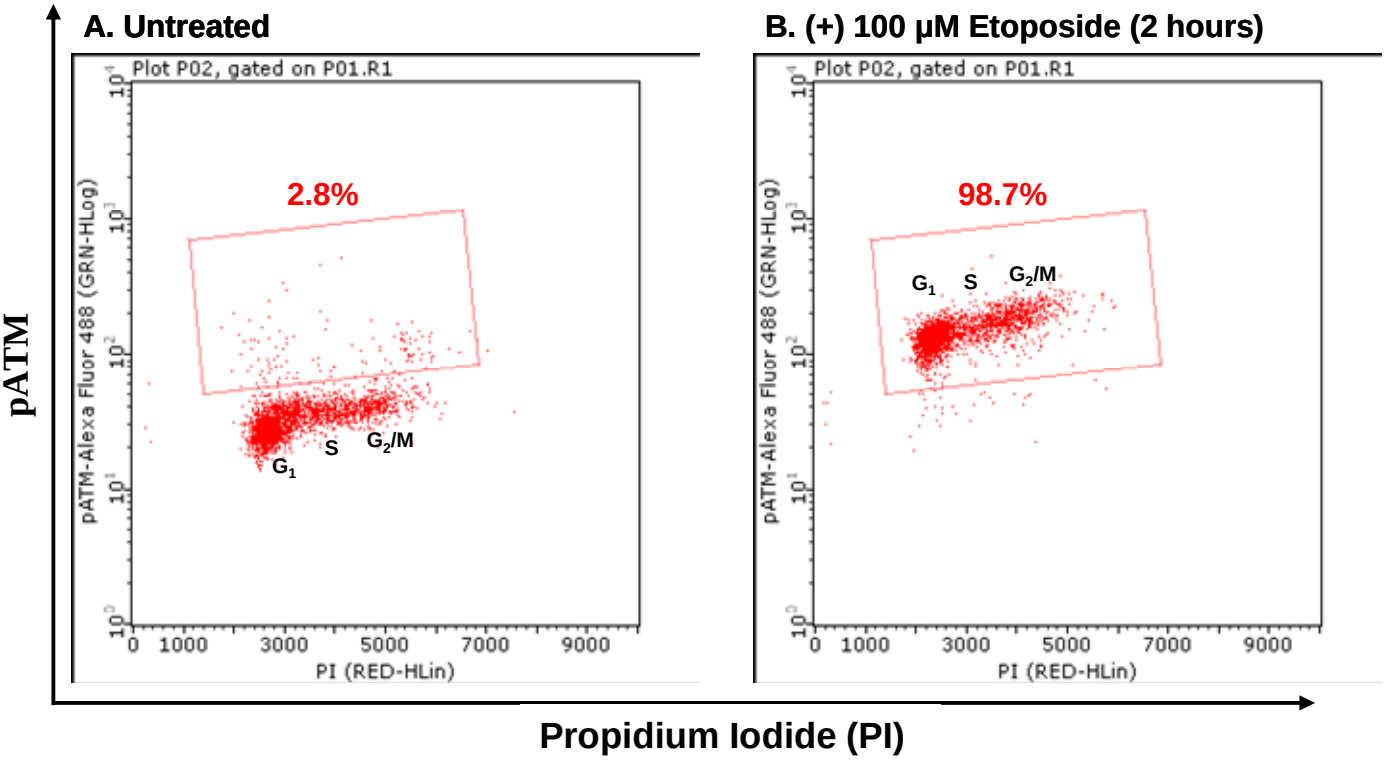

Figure 4. Etoposide induces DNA damage in all phases of the cell cycle DNA topoisomerase 2 inhibitors such as etoposide induced DNA damage in all phases of the cell cycle, with maximum in G1 phase. The phosphorylation of ATM on Ser1981 is maximal after treatment as seen in (B) at 98.7%, whereas in an untreated sample (A) only shows indicates 2.8%.

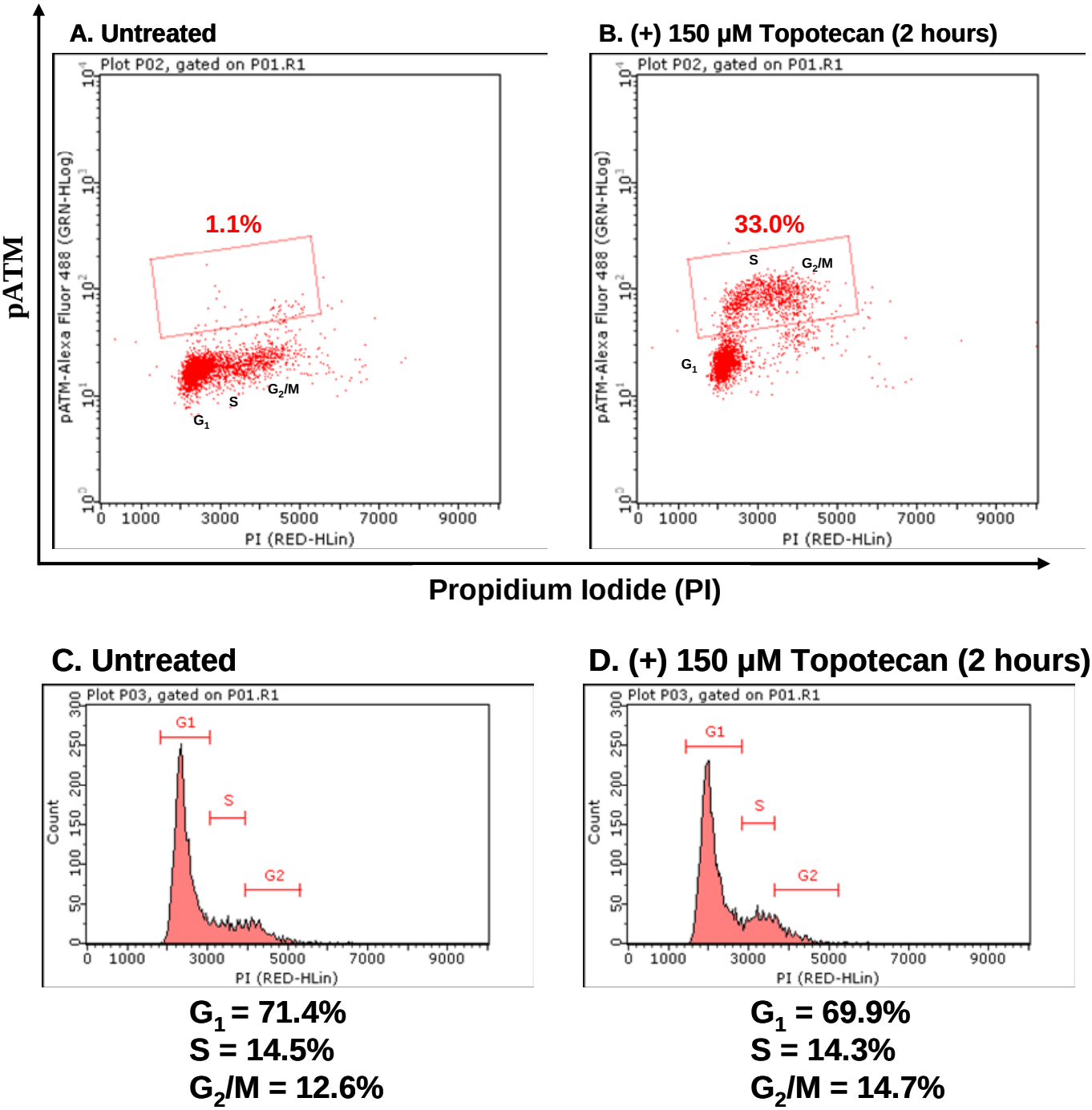

Figure 4. Bivariate analysis detecting DNA damage induced by Topotecan in relation to cell cycle positioning Topotecan is a topoisomerase1 inhibitor which causes DSBs in the late G1→ early S phases in the cell cycle, maximal at mid S phase. Treated HeLa cells show a marked increase in ATM phosphorylation, indicating DNA damage (B), when compared to the untreated sample (A), 33% versus 1%, respectively. Histograms C and D show cell cycle positioning between treated and untreated samples. Understanding where the first indication of DSBs takes place during the cell cycle can help researchers understand the mechanisms involved in cell cycle arrest and DNA repair at critical cell cycle checkpoints. This can serve useful application in the development of anti-neoplastic agents for cancer therapy.

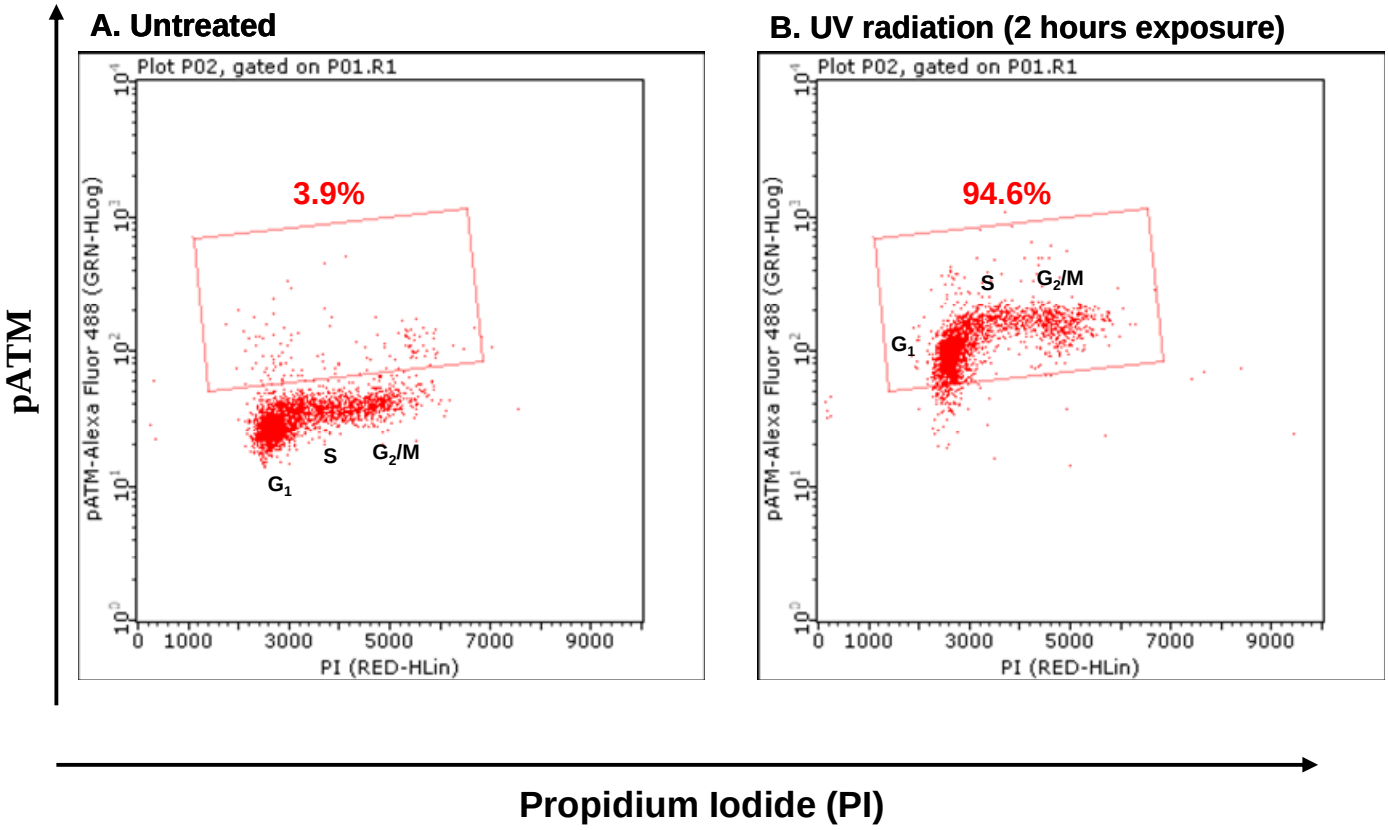

Figure 6. Ultra-violet light exposed HeLa cells to induce DNA damage and DSBs HeLa cells were either UV-irradiated (B) or left untreated (A) to cause DSBs. As indicated in the plots above, by UV treatment, cells showed a substantial increase in ATM phosphorylation (94.6%) compared to the untreated sample (3.9%). As noted by Tanaka et al. (2007), DSBs by X-radiation are the most critical in terms of their role in inducing reproductive cell death as well as delayed genomic instability, which may lead to tumorigenesis. Additionally, as noted by Zhao et al. (2010), exposure to UV light triggers the DNA damage response (DDR), seen as induction of ATM at late G1 → early S phase cells of the cell cycle.

Technical Hints

- Kit antibody, 5X Assay Buffer, and 1X Permeabilization Buffer should always remain at 2 - 8ºC, both prior and during use. For long term storage, Propidium Iodide can be stored at -20ºC. If keeping at -20ºC, avoid multiple freeze/thaw cycles.
- Mix samples thoroughly before use; avoid excessive bubbling.
- 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 to perform all steps in a smaller collection tube (e.g. micro-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 -20ºC, precipitate can form in the 10X wash buffer. Prior to use, place bottle in a 37ºC water bath, swirling the contents occasionally. If this does not remove the precipitate completely, allow 10X Wash Buffer to sit at room temperature overnight.
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.
Cellular Analysis	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.
Cellular Analysis	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*)

For further support, please contact Millipore's Technical services at +1(800) 437-7500

References

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7. Zhao, H., et al. (2010). Kinetics of the UV-Induced DNA Damage Response in Relation to Cell Cycle Phase. Correlation with DNA Replication. Cytometry Part A; 77A:285-293.

Related Products

1. FlowCellect™ Bivariate Cell Cycle Kit for DNA Replication Analysis (Catalog No. FCCH025102)
2. FlowCellect™ Bivariate Cell Cycle Kit for G2/M Analysis (Catalog No. FCCH025103)
3. FlowCellect ™ Multi-Color DNA Damage Response Kit (Catalog No. FCCH025104)
4. FlowCellect™ DNA Damage (Histone H2A.X) and Cell Cycle Analysis Kit (Catalog No. FCCH025142)
5. FlowCellect™ p38 Stress Pathway Activation Detection Kit (Catalog No. FCCS025132)
6. FlowCellect™ EGFR/MAPK Pathway Activation Kit (Catalog No. FCCS025101)
7. FlowCellect™ EGFR/STAT3 Pathway Activation Kit (Catalog No. FCCH025111)
8. FlowCellect™ PI3K-mTOR Signaling Cascade Mapping Kit (Catalog No. FCCS025210)
9. FlowCellect™ Multi-STAT Activation Profiling Kit (Catalog No. FCCS025550)
10. FlowCellect™ PI3K/MAPK Dual Pathway Activation and Cancer Marker Detection kit (Catalog No. FCCS025100)
11. FlowCellect™ PI3K Activation Dual Detection Kit (Catalog No. FCCS025105)
12. FlowCellect™ MAPK Activation Dual Detection Kit (Catalog No. FCCS025106)
13. FlowCellect™ EGFR RTK Activation Dual Detection Kit (Catalog No. FCCS025107)

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