FlowCellect™ Multi-Color DNA Damage Response Kit

25 Tests

Cat. No. FCCH025104

FOR RESEARCH USE ONLY
Not for use in diagnostic procedures.

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Introduction

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 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 (2-4). SMC1 is a member of the structural maintenance of chromosome family originally identified in yeast to be important for proper segregation of the chromosomes during mitosis (5). However, in mammalian cells exposed to IR, phosphorylation of SMC1 on sites S957 and S966 by ATM has been shown to be required for activation of the S-phase checkpoint and is important for protection against radiation induced cell death (3,6).

Phosphorylation of the histone variant, H2A.X, at serine 139 by ATM is an important indicator of DNA damage (7). 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 (8). γH2AX is also responsible for recruiting response proteins to the site of DNA damage (9).

Test Principle

Millipore's FlowCellect™ Multi-Color DNA Damage Response Kit is designed to enable a researcher a quick and easy way to detect the phosphorylation state of ATM, SMC1 and Histone H2A.X by flow cytometry. Millipore's FlowCellect™ Multi-Color DNA Damage Response Kit was developed and tested using the DNA damaging reagent, Etoposide, in HeLa cells as a model system but the kit can be used with other human cell lines to determine the effect of mechanical and chemical reagents that can induce DNA damage through the ATM dependent pathway.

Millipore's FlowCellect™ Multi-Color DNA Damage Response Kit contains sufficient reagents for 25 3-color samples. The kit includes three optimized fluorescently labeled antibodies and buffers necessary for cell preparation and analysis. Detailed assay instructions are included to assist in your analysis and to ensure that the correct cell concentration is obtained during acquisition of sample data.

Kit Components

1. Phospho Stain Buffer Pack: (Part No. CS203283) 1 box
2. 20X Anti-pSMC1 - AlexaFluor®488 Antibody: (Part No. CS204859) 1 vial containing 150 μL
3. 20X Anti-pATM - PE Conjugated Antibody: (Part No.CS204855) 1 vial containing 150 μL
4. 20X Anti-pHistoneH2A.X - PerCP Conjugated Antibody: (Part No. CS204852) 1 vial containing 150 μL

Materials Not Supplied

1. Guava Flow Cytometer
2. ViaCount™ reagent (Catalog No. 4000-0041)
3. Cell line of interest or HeLa cells for example protocol
4. Media for cell line of interest or MEM/EBSS – no L-glutamine with 10% FBS, 1% Non-essential amino acids, 1% Sodium pyruvate and 1% Penicillin/streptomycin/glutamine for example protocol.
5. HBSS
6. DMSO
7. Tissue culture instruments and supplies (including 37°C incubator, growth media, plates, detachment buffer, etc.)
8. Test tubes for sample and buffer preparation and storage.
9. Pipettors with corresponding tips capable of accurately measuring 1 – 1000 μL
10. Tabletop centrifuge capable of exceeding 2500 rpm.
11. Milli-Q™ Distilled Water or deionized water.
12. Etoposide (50mM stock in DMSO) or other treatment to induce DNA Damage

Precautions

- Some assay components included in the kit may be harmful. The fixation buffer contains formaldehyde. Please refer to the MSDS sheet for specific information on hazardous materials.
- All fluorochrome conjugated antibodies are light sensitive and must be stored in the dark at 4°C.
- During storage and shipment, small volumes of product will occasionally become entrapped in the seal of the product vial. For maximum recovery of product, centrifuge vial briefly prior to removing cap.
- Do not use reagents beyond 6 months after date of receipt.

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Storage

Upon receipt, all antibodies and buffers should be stored at 4°C. ***Caution:*** *Fluorochrome conjugated antibodies should always be stored at 4°C. Any deviation in temperature for long periods of time may compromise the antibodies performance.* Discard any remaining reagents after 6 months.

Example Protocol

Note*: This assay protocol has been optimized for HeLa human cervical carcinoma (ATCC #CCL-2) cells treated with etoposide. However, this kit is suitable for flow cytometric analysis of a variety of human cell types and treatments but must be confirmed by the end user.*

Cell Preparation for Etoposide treatment

1. Seed $10X10^6$ cells into 2 T-75 flasks ($5X10^6$ cells/flask).
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. Incubate the flasks in a 37°C/5% CO_2 incubator overnight.
5. Next day in the morning, replace the media in both flasks with 20ml of fresh media.
6. To the **Treated** flask and add 40 µL of 50 mM Etoposide (in DMSO) for a final concentration of 100 µM. To the **Untreated** flask and add 40 µL of DMSO.
7. Incubate the flasks in a 37°C/5% CO_2 incubator for 24 hours.
8. After 24 hours incubation, aspirate the media and rinse both T75 flasks with 5 ml HBSS.
9. Add 1 mL accutase in DPBS, 0.5 mM EDTA (Millipore# SCR005) to each flask and incubate at 37°C for 5 min; if necessary, tap gently to dislodge cells.
10. 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.**
11. Count cells using ViaCount™ reagent.

Preparation of Buffers

Note: *Prepared 1X Fixation Buffer is stable up to one month if stored at 4ºC, therefore should only be made before starting assay. Prepared 1X Wash and Assay Buffers are stable up to six months if stored at 4ºC, and can be prepared when you receive the kit or when you start the first assay.*

Buffer Preparation for 5 untreated and 5 treated samples (Scale up or down as necessary).

1. Make 10 mL of Wash Buffer. Add 1 mL of 10X Wash Buffer and 9 mL of deionized water.

2. Make 10 mL of 1X Fixation buffer. Add 1mL of 10X Wash Buffer, 2.5 mL of Fixation buffer and 6.5 mL of deionized water.

3. Make 30 mL of 1X Assay Buffer. Add 6 mL of 5X Assay Buffer and 24 mL of deionized water.

4. Place Permeabilization Buffer on ice.

Example Cell Staining Protocol:
Note**:** *You will need 0.5X10^6 cells for one test on Guava cytometer and 1X10^6 cells for one test on other flow cytometers.*

1. Place **Treated** cells into a conical labeled **Treated** and place **Untreated** cells into a conical labeled **Untreated**. (All steps from this point will be performed for both samples)

2. Spin down cells at 2500 rpm (600Xg) for 3 minutes and discard media.

3. Resuspend cells in 1 mL of 1X Wash Buffer/1X10^6 cells by gently pipetting up and down.

4. Spin down cells at 2500 rpm (600Xg) for 3 minutes and discard buffer.

5. Resuspend cells in 1 mL of 1X Fixation Buffer/1X10^6 cells by gently pipetting up and down and incubate for 20 minutes at room temperature.

6. Spin down cells at 2500 rpm (600Xg) for 3 minutes and discard buffer.

7. Resuspend cells in 1 mL 1X Assay Buffer/1X10^6 cells by gently pipetting up and down.

8. Spin down cells at 2500 rpm (600Xg) for 3 minutes and discard buffer.

9. Resuspend cells in 1 mL 1X Permeabilization Buffer/1X10^6 cells by gently pipetting up and down.

10. Incubate samples on ice for 5 minutes.

11. Spin down cells at 2500 rpm (600Xg) for 3 minutes and discard buffer.

12. Resuspend cells in 0.5 mL 1X Assay Buffer/1X10^6 cells by gently pipetting up and down.

13. Aliquot 0.5X10^6 cells (Guava) or 1X10^6 cells (Other) into individual microfuge tubes. **Untreated** cells will be labeled tube 1, 3, 5, and 7. **Treated** cells will labeled tube 2, 4, 6 and 8. Keep any unused cells on ice.

14. Spin down cells at 2500 rpm (600Xg) for 3 minutes and discard buffer.

15. Resuspend cells in tubes 1-6 in 95 μL(Guava) or 90 μL(Other) of 1X Assay Buffer by pipetting up and down. Resuspend cells in tubes 7 and 8 in 85 μL(Guava) or 80 μL(Other) of 1X Assay Buffer by pipetting up and down.

16. Add 5 μL(Guava) or 10 μL(Other) of 20X antibody to tubes using the flowing chart.

Tube #	Description
1	Untreated cells stained with pSMC1-AlexaFluor®488
2	Treated cells stained with pSMC1-AlexaFluor®488
3	Untreated cells stained with pATM-PE
4	Treated cells stained with pATM-PE
5	Untreated cells stained with pHistoneH2A.X-PerCP
6	Treated cells stained with pHistoneH2A.X-PerCP
7	Untreated cells stained with all three antibodies
8	Treated cells stained with all three antibodies

17. Incubate tubes in the dark on ice for 60 minutes.

18. Add 900 μL of 1X Assay Buffer to each tube then pellet the cells at 2500 rpm for 3 minutes and discard buffer.

19. Resuspend cells in 500 μL(Guava) or 1 mL(Other) of 1X Assay Buffer.

20. Transfer cells to a sample tube according to the following chart and analyze on a Flow Cytometer:

Sample	Instructions
1	250 μL of Tube 1 and 250 μL of Tube 2.
2	250 μL of Tube 3 and 250 μL of Tube 4.
3	250 μL of Tube 5 and 250 μL of Tube 6.
4	500 μL of Tube 7
5	500 μL of Tube 8

Note: Samples 1, 2, and 3 are mixtures of treated and untreated cells which are used for adjusting compensation. Once the initial compensation for a given cell type/treatment has been performed the settings file can be saved and used for subsequent data collection without the need for samples 1, 2, and 3.

Sample Data

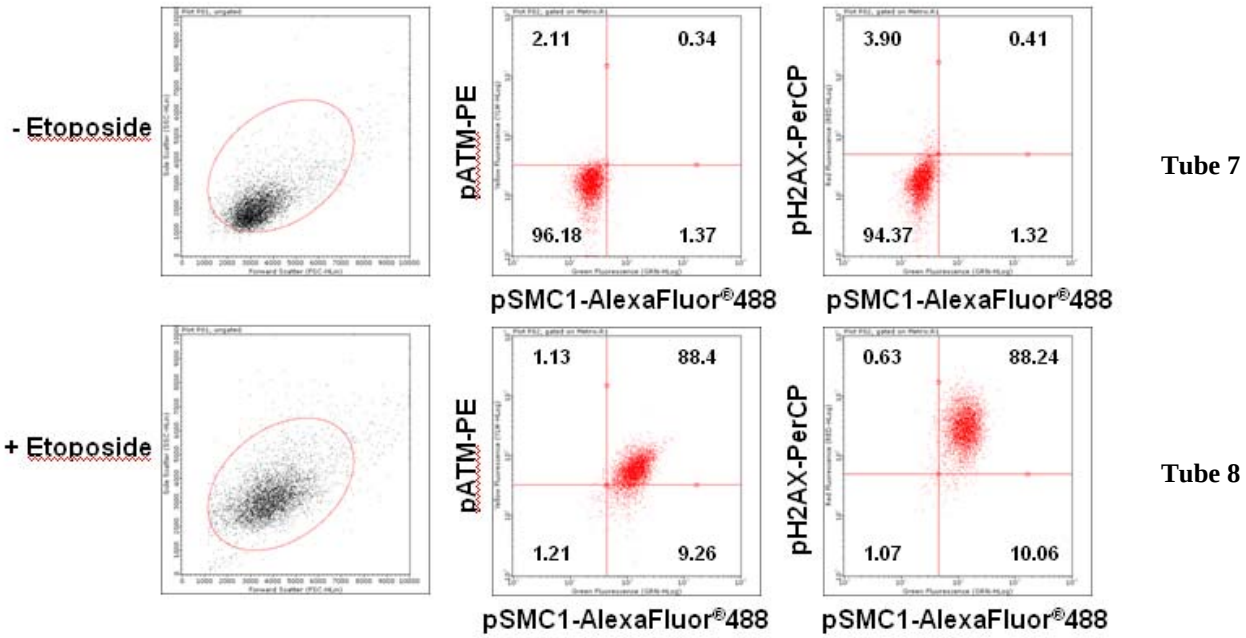

Figure 1: Representative data from HeLa cells with Etoposide (bottom panels of plots) and without Etoposide (top panel of plots). Gating on the live cells (FSC vs. SSC) is shown in the first column and the dot plot data for the green, yellow and red fluorescence channels are shown in the second and third column.

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

Potential Problem	Experimental Suggestions
Acquisition rate decreases dramatically Instrument clogging Too many cells	• Too many cells per microliter- Decrease the number of cells by diluting sample to 300 – 500 cells per microliter. The Guava flow cytometer gives the most accurate data when the flow rate is less 500 cells per microliter. • Run three Quick Cleans to rinse out the flow cell. This procedure can be performed during or after an assay. This will wash away any material forming within the glass capillary walls.
Too few cells	• Spin down cells and resuspend in a smaller volume. The assay instructions are optimized to give you a range of cells between 100-500 cells/μL in the final sample volume. However, cell loss is common during washing steps in the assay procedure. A substantial decrease in cell numbers can lead to difficulty in adjusting settings. Make sure to leave the cell pellet intact when discarding buffer. If the cells are not generating a compact pellet after centrifugation, increase the time to 5 minutes and/or increase the speed by 500 rpm until a compact and visible cell pellet forms.
Background staining 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 changes in culture conditions or viability can influence experimental results. • When using the Guava flow cytometer for flow analysis, make sure that a quality check on the instrument (e.g. calibration) is performed on a daily basis prior to use.
Staining is weak	• Some cell lines may require higher concentrations of fixation buffer to stain correctly. Use the Fixation Buffer at 2X rather than 4X.
Staining is weak	• Some cell lines may require higher concentrations of conjugated antibodies. Use the antibodies at 10X rather than 20X.
Staining is too bright	• Some cell lines may require lower concentrations of conjugated antibodies. Use the antibodies at 40X rather than 20X.

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

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