DNA Damage Assay
(Fluorescent)

(version A)

Catalog No. 18030

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TABLE OF CONTENTS **Page**

Introduction

Active Motif's DNA Damage Assay is a simple, fast and accurate 2-color fluorescent assay for the induction of double-stranded DNA breaks and apoptosis. When cell cultures or animals are exposed to ionizing radiation or certain treatment compounds, double-stranded DNA breaks are created that rapidly result in the phosphorylation of the histone variant H2AX at serine 139[1,2]. Because H2AX phosphorylation at serine 139 correlates very closely with each double-stranded DNA break, phospho-H2AX is a sensitive marker for DNA damage and can be used as a marker for apoptosis.

The assay utilizes a highly specific DNA damage and apoptosis marker antibody, anti-phospho-Histone H2AX (Ser 139), which is detected by a fluorescent Chromeo™ 488-labeled secondary antibody. Cells are grown and treated in a 96-well plate, then fixed and incubated with the phospho-H2AX apoptosis marker antibody, which is subsequently detected with the Chromeo 488 secondary. Lastly, the cells are washed and stained with propidium iodide and the plate is read on a fluorescent plate reader.

The kit contains all the necessary reagents for two 96-well plates including apoptosis marker antibody, fluorescent secondary antibody, propidium iodide and etoposide, which is a compound that causes high levels of DNA damage, so can be used in positive control assay wells. This assay is well suited for high-throughput and high-content fluorescent scanning applications.

product	format	catalog no.
DNA Damage Assay (Fluorescent)	2 x 96 rxns	18030

Measuring the Percent Induction of Apoptosis

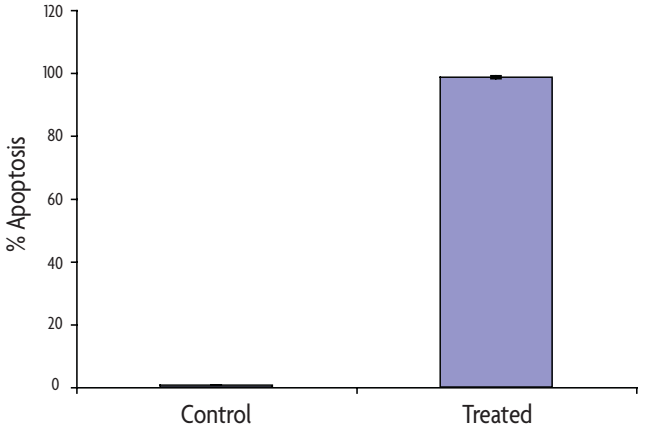

Figure 1: Percent induction of apoptosis in HeLa cells.
Levels of phospho-H2AX were measured in untreated (control) HeLa cells or in cells treated for six hours with etoposide. Averages of quadruplicate wells are shown.

Scatter Plot Analyses of Phospho-H2AX vs. Propidium Iodide

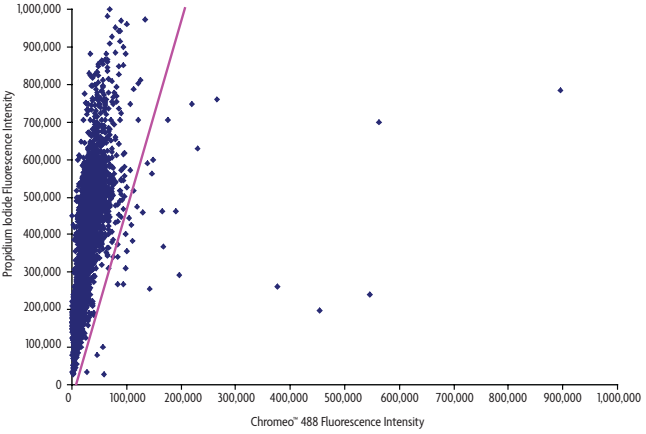

Figure 2: Single cell analysis of phospho-H2AX antibody detected with Chromeo™ 488 Goat anti-rabbit IgG.
Untreated HeLa cells were incubated with phospho-H2AX antibody. The majority of the cell population is to the left of the dividing line and visualized in the propidium iodide channel indicating a normal cell population. Few cells are stained with the phospho-H2AX antibody. Scatter plot analysis was performed using the IsoCyte™.

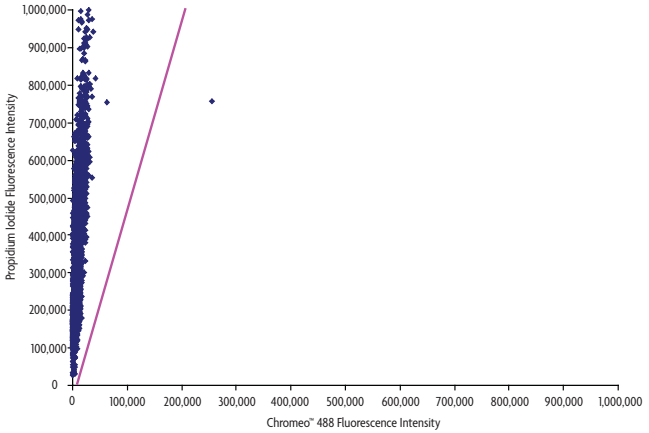

Figure 3: Single cell analysis of control HeLa cells treated only with propidium iodide.
Untreated HeLa cells were incubated with propidium iodide. All cells are visualized in the propidium iodide channel, indicating normal cell populations. Cell populations were quantified using the IsoCyte™.

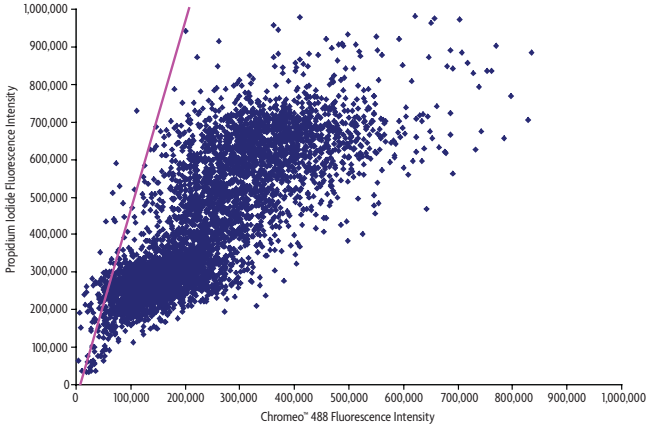

Figure 4: Single cell analysis of etoposide treated HeLa cells.
HeLa cells were treated with etoposide for six hours. An increase in the amount of phospho-H2AX staining was detected with the Chromeo™488 Goat anti-Rabbit IgG as observed using the IsoCyte™. The resulting shift of the population to the right of the dividing line indicates a high degree of DNA damage in these cells.

Kit Components and Storage

Please store each component at the temperature indicated in the table below.

Reagents	Quantity	Storage / Stability
Anti-phospho-H2AX (S139)	40 µl	-20°C
Chromeo™ 488 Goat anti-Rabbit IgG	20 µl	4°C
Propidium Iodide	40 µl	4°C
RNase A (lyophilized)	reconstitute with 110 µl H_2O	4°C
Etoposide	10 µl	-20°C

Additional materials required

- 100% ice cold methanol
- 5% BSA in PBS
- Sterile H_20
- 96-well, black-walled, clear-bottom plastic plates such as Corning catalog number 3904
- Fluorescent imaging system capable of 488 nm excitation
- Multi-channel pipettor

Alternative DNA damage-inducing agents

- Mitomycin C (forms DNA crosslinks)
- Aphidicolin (DNA polymerase inhibitor; halts the cell in G1/early S phase)
- Camptothecin (potent inhibitor of topoisomerase I, a molecule required for DNA synthesis. Camptothecin has been shown to induce apoptosis in a dose-dependent manner *in vitro)*
- Doxorubicin (inhibits topoisomerase II, binds to nucleic acids and inhibits nucleic acid synthesis)

Reagent Preparation

Preparation of Etoposide Solution

Prepare a 1:500 dilution (final concentration will be 100 µM) of etoposide in the standard cell culture medium being used. Remove the existing medium in the wells and apply 200 µl of the Etoposide Solution. Sufficient etoposide is provided to treat two columns (16 wells) of a 96-well plate.

Note: In some experiments, small increases in signal have been observed in negative control wells that are immediately adjacent to the etoposide-treated wells. Thus, the etoposide is potent enough that it may in some instances effect wells to which it has not even been added. Keep this in mind when deciding which wells in your 96-well plate will be used for your test samples and controls.

Preparation of Propidium Iodide/RNase A Solution

Reconstitute the RNase A with 110 µl sterile H_2O. The concentration of this RNase A stock solution will be 10 mg/ml. It will be used at a final concentration of 10 µg/ml (1:1000 dilution) in PBS.

The stock solution of propidium iodide is provided at a concentration of 1 mg/ml. It will be used at a final concentration of 1 µg/ml (1:1000 dilution) in PBS.

The volume of Propidium Iodide/RNase A Solution required to treat one 96-well plate is:

200 µl x 96 = 19,200 µl

To prepare sufficient Propidium Iodide/RNase A Solution for one 96-well plate, add 20 µl propidium iodide (for a final concentration of 1 µg/ml) and 20 µl RNase A to 20 ml pre-warmed (37°C) PBS. Add 200 µl to each well. Incubate at 37°C for 30 minutes, then image the cells. See Step 3 for details.

Note: It is not necessary to wash the cells following the addition of the Propidium Iodide/RNase A Solution.

Protocol

Step 1: Culture, Fix and Block the Cells

1. Seed the cells in a plastic, black-walled, clear-bottom 96-well plate so that they will be at the desired confluency at the time of fixing, after they have been treated as desired. For single cell IsoCyte™ experiments, 4000 HeLa cells per well is suitable. For other plate readers, a higher density may be required (20,000 cells per well).
2. Grow and treat cells as desired. For the positive control Etoposide Solution, it is recommended to induce apoptosis the day after the cells were seeded. Add 200 µl per well for 6 hours at 37°C is . The concentration of etoposide can be reduced if a longer treatment with test compounds is desired.

   Note: Depending on experimental design, some wells should be incubated with the positive control Etoposide Solution, some wells should be left untreated and the remaining wells can be treated with your test compounds. Background staining can be reduced by washing all cells with room temperature PBS then applying fresh cell culture medium to control wells or etoposide-containing media to positive control wells.

3. Fix cells by replacing the growth medium with 100 µl of 100% ice-cold methanol for 10 minutes.
4. Remove methanol and wash the cells 3 times with 200 µl PBS. Each wash step should be performed for 5 minutes.
5. Remove PBS, add 100 µl BSA (5%) in PBS and incubate for 1 hour at room temperature.

Step 2: Binding of Primary and Secondary Antibodies

6. Remove the BSA/PBS solution and wash cells 3 times with 200 µl PBS.
7. Remove PBS and add 50 µl of primary antibody diluted 1:250 in BSA/PBS. Add 50 µl BSA/PBS only for the negative control wells.
8. Seal the plate with sealing tape, cover with lid and incubate overnight at 4°C. Make sure the plate is level and that each well is tightly sealed with the tape to prevent evaporation.

   Note: The primary antibody can also be applied to the cells for 1 hour at room temperature.

9. Remove primary antibody, wash cells 3 times for 5 minutes each with 200 µl PBS.
10. Remove the PBS, add 100 µl diluted secondary antibody (1:1000), cover plate with tissue culture plate lid or sealing tape, and incubate 1 hour at room temperature.

Step 3: Adding Nuclear Marker and Scanning the Plate

11. Remove secondary antibody, wash cells twice for 5 minutes each with 200 µl PBS.
12. Add 200 µl of the Propidium Iodide/RNase A Solution prepared above.
13. Wait 30 minutes for propidium iodide to stain the cells, then scan the plate.

    Note: Propidium iodide may be omitted from the protocol if the plate is to be read on a standard fluorescent plate reader.

References

1. Rogaku, E.P. *et al.* (1998) *J. Biol. Chem.* 273(10): 5858-5868.
2. Bewersdorf, J. *et al.* (2006) *PNAS* 103:18137.

Appendix

Section A. Troubleshooting Guide

Problem/question	Recommendation
No H2AX signal is observed.	Check that all the reagents have been added in the correct order. Use Western blotting to confirm that the cells contain a detectable level of phospho H2AX. Check that cells adhered to the plate. If not, try coating the plate with poly-lysine prior to seeding the cells.
No increase in DNA damage is seen after treatment with etoposide, or the increase is not satisfactory.	Treat cells for a longer time period, change cell model or increase concentration of etoposide.
DNA damage levels appear high in the untreated control cells.	This may be due to a high number of dying cells in the control well. Check that CO_2 levels in the incubator are optimal. Reduce the number of cells plated as over-confluency can lead to a high level of staining in control wells. If cell passage is high, try seeding cells of a lower passage number. Try increasing the number of wash steps to reduce non-specific staining.

Section B. IsoCyte Imaging and Data Analysis

IsoCyte scanning conditions

Use Ch1 (510-540 nm emission filter) to image the Chromeo™ 488 Goat anti-Rabbit IgG-labeled phospho-H2AX (Ser 139) and Ch3 (600 LP or 650 LP filter) to image the propidium iodide.

1. Turn on the scanner using the On/Off (I/O) switch located on the left side of the instrument. Wait 5 minutes for the laser to warm up.
2. Start the BBIsoCyte software by double-clicking on the icon on the desktop.
3. Click on the blue arrow-shaped icon on the tool bar to eject the plate holder.
4. Insert plate and click icon again to close.
5. Open Method.
6. Select the wells that are to be scanned. The wells will be highlighted in blue if they have been selected.
7. Click on the Scan icon.
8. When the scan has finished, save the data file.

Section C. Related Products

Fluorescent Dyes	Format	Catalog No.
Chromeo™ 488 Carboxylic Acid	1 mg	15510
	5 mg	16510
Chromeo™ 488 NHS-Ester	1 mg	15511
	5 mg	16511
Chromeo™ 488 Biotin	1 mg	15512
	5 mg	16512
Chromeo™ 488 Streptavidin	1 mg	15513
	5 mg	16513
Chromeo™ 494 Carboxylic Acid	1 mg	15110
	5 mg	16110
Chromeo™ 494 NHS-Ester	1 mg	15111
	5 mg	16111
Chromeo™ 494 Biotin	1 mg	15112
	5 mg	16112
Chromeo™ 494 Streptavidin	1 mg	15113
	5 mg	16113
Chromeo™ 546 Carboxylic Acid	1 mg	15210
	5 mg	16210
Chromeo™ 546 NHS-Ester	1 mg	15211
	5 mg	16211
Chromeo™ 546 Biotin	1 mg	15212
	5 mg	16212
Chromeo™ 546 Streptavidin	1 mg	15213
	5 mg	16213
Chromeo™ 642 Carboxylic Acid	1 mg	15310
	5 mg	16310
Chromeo™ 642 NHS-Ester	1 mg	15311
	5 mg	16311
Chromeo™ 642 Biotin	1 mg	15312
	5 mg	16312
Chromeo™ 642 Streptavidin	1 mg	15313
	5 mg	16313

Fluorescent Secondary Antibodies	Format	Catalog No.
Chromeo™ 488 Goat anti-Mouse IgG	1 mg	15031
Chromeo™ 488 Goat anti-Rabbit IgG	1 mg	15041
Chromeo™ 494 Goat anti-Rabbit IgG	1 mg	15042
Chromeo™ 546 Goat anti-Mouse IgG	1 mg	15033
Chromeo™ 546 Goat anti-Rabbit IgG	1 mg	15043
Chromeo™ 642 Goat anti-Mouse IgG	1 mg	15034
Chromeo™ 642 Goat anti-Rabbit IgG	1 mg	15044

Mitosis Assays	Format	Catalog No.
Mitotic Index Assay Kit (Fluorescent)	5 x 96 rxns	18020
Mitotic Assay Kit (Color)	2 x 96 rxns	18021
Mitotic Assay Kit (Chemi)	2 x 96 rxns	18022

Cell Viability Assay	Format	Catalog No.
ToxCount™	20 x 96 rxns	18010

Technical Services

If you need assistance at any time, please call Active Motif Technical Service at one of the number listed below.

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