INSTRUCTIONS

Thermo SCIENTIFIC

Cellomics® Phospho-H2AX Activation Kits

For High-Content Screening

1991.0

Number	Description
8402901	**Phospho-H2AX Activation Kits,** sufficient materials for 1 × 96 wells
8402902	**Phospho-H2AX Activation Kits,** sufficient materials for 5 × 96 wells

Kit Contents	8402901	8402902
Phospho-H2AX Primary Antibody	6 µl	30 µl
DyLight™ 549 Conjugated Goat Anti-Mouse IgG	30 µl	72 µl
Hoechst Dye	30 µl	30 µl
Wash Buffer (10X Dulbecco's PBS)	100 ml	100 ml
Wash Buffer II (10X Dulbecco's PBS with 1% Tween®-20)	100 ml	100 ml
Permeabilization Buffer (10X Dulbecco's PBS with 1% Triton® X-100)	100 ml	100 ml
Blocking Buffer (10X)	85 ml	85 ml
Thin Plate Seal Assembly	7/pack	7/pack

Storage: Upon receipt immediately store the Phospho-H2AX Primary Antibody at -20°C. Store all other components at 4°C. Keep vials containing the fluorescent antibody and Hoechst Dye solutions protected from light. Allow buffers to warm to room temperature before use. See the **Solution Preparation** section for storage and stability of prepared solutions.

Warning: Please completely read these instructions and the accompanying material safety data sheets before using this product. The Cellomics Reagents are not for diagnostic use in humans or animals.

Introduction

The Cellomics Phospho-H2AX Activation Kit contains optimized reagents for the detection and quantitation of phosphorylated H2AX (Ser139) in the nucleus. The kit contains a primary monoclonal antibody that detects only the phosphorylated form of human H2AX, a goat anti-mouse secondary antibody conjugated to DyLight 549 Fluorophore and various other reagents and buffers required for immunofluorescence staining for high-content screening (HCS) assays.

The nucleosome is made of four core histone proteins (H2A, H2B, H3 and H4). H2AX belongs to a H2A family of histones. DNA damage induction by various agents leads to rapid phosphorylation of H2AX at Ser139 (also known as Gamma H2AX) by ATM, ATR or DNA protein kinase leading to the formation of DNA foci at the site of DNA double-strand breaks (DSBs). Phosphorylated H2AX helps in recruiting the proteins responsible for double-strand break repair.[1-3]

Phosho-H2AX in the nucleus of A549 cells treated with etoposide was assayed using the Cellomics Phospho-H2AX Activation Kit, the Cellomics ArrayScan® HCS Reader[4] (Figure 1) and the Compartmental Analysis BioApplication Software Module. Induction of DNA damage by etoposide leads to phosphorylation of H2AX. The output parameter for this assay is the nuclear intensity of phosphor-H2AX staining after treatment. Cells labeled using this kit also can be imaged by fluorescence or confocal microscopy.

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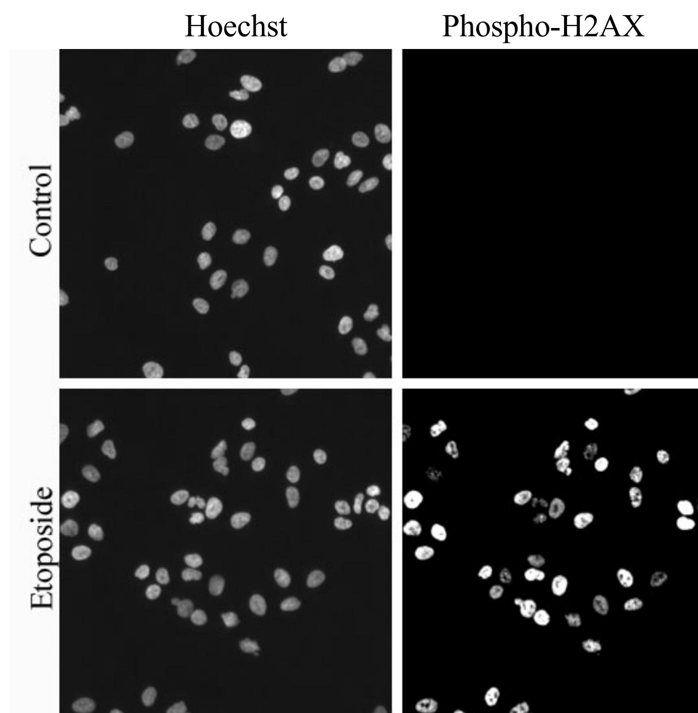

Figure 1. Staining of Phospho-H2AX in A549 cells. Cells were treated with vehicle (control; 0.1% DMSO in media) or with 50 µM etoposide for 1 hour, stained according to the kit protocol, and imaged using a Cellomics ArrayScan HCS Reader.

Additional Materials Required

- Paraformaldehyde (16%) (Thermo Scientific 16% Formaldehyde, Product No. 28906)
- Packard View 96-well Microplates (e.g., Perkin-Elmer, Product # 6005182)
- Positive control compound such as etoposide (Sigma Aldrich # E1383)
- Fetal bovine serum

Cell Preparation Information

- This protocol is optimized for A549 cells (American Type Culture Collection #CCL-185). This kit also effectively stains the following cell types: HeLa and HepG2.
- For routine culture of cells, use F12K medium supplemented with 10% fetal bovine serum, 100 units/ml penicillin and 100 µg/ml streptomycin (F12K complete medium).
- For phospho-H2AX detection, harvest cells by trypsinization, dilute into F12K complete medium and determine cell density. Dilute cells to 7.5×10^4 cells/ml in F12K complete medium and add 100 µl of the cell suspension per well of a 96-well microplate to achieve the recommended plating density of 7,500 cells/well.
- Split cells when they reach 90% confluence at a dilution of 1:3. Use cells at a passage number ≤ 20.
- Incubate cells overnight at 37°C in 5% CO_2 before drug treatment.

Phospho-H2AX Activation Kit Protocol

A. Solution Preparation (per 96-well plate)

1X Wash Buffer	Add 20 ml 10X Wash Buffer to 180 ml ultrapure water for a final volume of 200 ml. Store buffer at 4°C for up to 7 days.
1X Wash Buffer II	Add 6 ml of 10X Wash Buffer II to 54 ml ultrapure water for a final volume of 60 ml. Store buffer at 4°C for up to 7 days.
Fixation Solution	Add 3 ml of 16% paraformaldehyde solution to 9 ml of 1X Wash Buffer just before use.
1X Permeabilization Buffer	Add 1.5 ml of 10X Permeabilization Buffer to 13.5 ml of the 1X Wash Buffer. Store this buffer at 4°C for up to 7 days.
1X Blocking Buffer	Add 5 ml of 10X Blocking Buffer to 45 ml of 1X Wash Buffer for a final volume of 50 ml. Add a final concentration of 2% fetal bovine serum (FBS). Store this buffer at 4°C for up to 7 days.
Primary Antibody Solution	Add 6 µl of Phospho-H2AX Primary Antibody to 6 ml of 1X Blocking Buffer. Prepare solution just before each assay.
Secondary Antibody/ Staining Solution	Add 0.6 µl of Hoechst Dye and 12 µl of the DyLight 549 Goat Anti-Mouse secondary antibody to 6 ml of 1X Blocking Buffer. Prepare solution just before each assay.

B. Procedure

1. Prepare 2X solution of etoposide (100 µM) and add 100 µl to the cells. Incubate cells for 1 hour at 37°C.
2. Aspirate culture medium and add 100 µl of Fixation Solution to each well. Incubate plate in a fume hood at room temperature for 15 minutes.
3. Aspirate Fixation solution, and wash plate twice with 100 µl/well 1X Wash Buffer.
4. Aspirate Wash Buffer and add 100 µl/well of 1X Permeabilization Buffer and incubate for 15 minutes at room temperature.
5. Aspirate Permeabilization Buffer and wash plate twice with 100 µl/well of 1X Wash Buffer.
6. Aspirate Wash Buffer, add 100 µl/well of the 1X Blocking Buffer and incubate at room temperature for 15 minutes.
7. Aspirate Blocking Buffer and add 50 µl/well Primary Antibody Solution. Incubate for 1 hour at room temperature.
8. Aspirate Primary Antibody Solution and wash plate twice with 100 µl/well 1X Wash Buffer II.
9. Aspirate Wash Buffer II and wash plate twice with 100 µl/well 1X Wash Buffer.
10. Aspirate Wash Buffer and add 50 µl/well of Secondary Antibody/Staining Solution. Incubate for 45 minutes at room temperature protected from light.
11. Aspirate Staining Solution and wash plate twice with 100 µl/well of 1X Wash Buffer II.
12. Aspirate Wash Buffer II and wash plate twice with 100 µl/well of 1X Wash Buffer.
13. Aspirate Wash Buffer and replace with 200 µl/well of 1X Wash Buffer.
14. Seal plate and evaluate on the ArrayScan HCS Reader. Store plates at 4°C.

Additional Information

A. Dose Response Curve

A549 cells were treated with different doses of etoposide for 1 hour as described in the procedure, and phospho-H2AX intensity was measured. The feature plotted is the Mean_CircAvgInt, which is the average nuclear intensity (Figure 2).

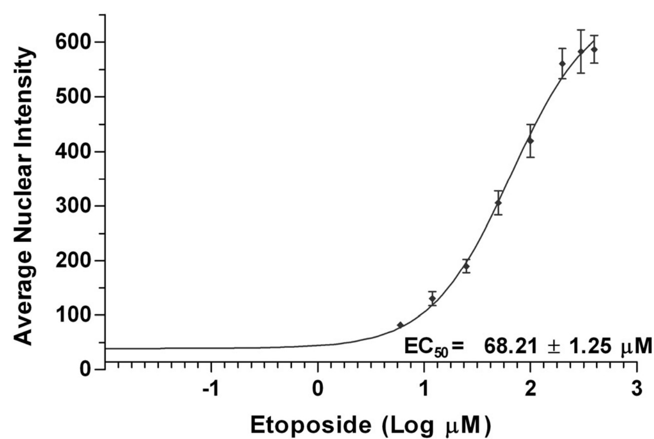

Figure 2. Dose response curve for etoposide-treated A549 cells. Data represents mean ± SD from three plates (eight wells per 96-well plate per dose of etoposide).

B. Performance Robustness

The robustness of the kit was ascertained by determining the Z′ for the average intensity of phosphor-H2AX in non-treated (vehicle) and etoposide- (50 µM for 1 hour) treated cells.[4,5] The Z′ for phospho-H2AX activation was calculated using three plates of A549 cells treated identically and was 0.68 ± 0.06.

DMSO tolerance: The assay performance using these kits was robust when compounds were added in DMSO up to a maximum concentration of 1% DMSO.

C. Microscope Information

Cells prepared and labeled according to these instructions can be used and analyzed by fluorescence microscopes using the appropriate filter set(s) or confocal microscopy. Optimization may be required when using slides, coverslips or multi-well chamber slides. Use image-processing software to quantify the targets. The approximate absorption/emission maxima of the fluorescent dyes are as follows:

DyLight 549 Conjugates = 550/568 nm

Hoechst Dye = 350/461 nm

D. Recommendations for Automation

- Plating Cells: To improve the uniformity and throughput of plating cells, use a liquid handling system such as a Thermo Scientific Multidrop® Combi or WellMate® Dispensers.
- Dead Volumes: Every piece of automation instrumentation has a non-recoverable dead volume associated with it. Be aware of these dead volumes, priming volumes and rinsing volumes when calculating your reagent requirements.
- Nonspecific Binding: Because of the potential of reagent interaction with large surface areas inherent to tubing, syringes and peristaltic pumps, pre-priming with reagents or pre-coating with protein blockers may be warranted.
- Mixing: Gentle mixing may be required when adding a DMSO-based solution to keep overly concentrated solutions from lying on top of the cell layer. Be careful not to dislodge cells or beads during mixing procedures.
- Cell Washing: Use an automated plate washer designed to gently wash attached cells. Be careful not to dislodge cells or beads during cell washing.
- Incubation: Minimize the time when plates with live cells are out of a controlled CO_2 environment. For best results, use an automated incubator to deliver plates to a pipetting deck.

- Exposure: Minimize operator exposure to fixative by some form of containment. Some reagents and compounds are light-sensitive; be aware of these constraints when scaling up for an automated run.
- Adapting to other plate formats: When using different plate types, adjust reagent volumes as needed. Some suggested starting volumes are listed in Table 1.

Table 1. Suggested volumes to use for different cell culture plates.

Kit Component	96-Well Plates (µl/well)	384-Well Plates (µl/well)	24-Well Plates (µl/well)
Fixation Solution	100	25	400
1X Wash Buffer	100	25	400
Wash Buffer II	100	25	400
1X Permeabilization Buffer	100	25	400
1X Blocking Buffer	100	25	400
Antibody Solution	50	12.5	200
Staining Solution	50	12.5	200
1X Wash Buffer (final wash)	150	37.5	200

Compatible BioApplication Software Modules

S50-0001-1 or S50-2001-1 **Cytoplasm to Nucleus Translocation BioApplication**

S50-5019-1 or S50-2019-1 **Molecular Translocation BioApplication**

S50-5011-1 or S50-2011-1 **Target Detection BioApplication**

S50-5017-1 or S50-2017-1 **Compartmental Analysis BioApplication**

References

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2. Rogakou E.P., *et al.* (1999). Megabase chromatin domains involved in DNA double-strand breaks *in vivo*. *J Cell Biol* **146:**905-16.
3. Burma S., *et al*. (2001). ATM phosphorylates histone H2AX in response to DNA double strand breaks. *J Biol Chem* **276:**42462-7.
4. Taylor, D.L., *et al.* (2007). High content screening: A powerful approach to systems cell biology and drug discovery. *Method Mol Biol* **356**. Humana Press, Totowa, N.J.
5. Zhang, J.H., *et al.* (1999). A simple statistical parameter for use in evaluation and validation of high throughput screening assays. *J Biomol Screen* **4**:67-73.

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Thermo Scientific Cellomics Reagent Kits are developed and manufactured at the same Thermo Fisher Scientific Inc. facility as Pierce Protein Research Products and are supported by Pierce Technical Support (see contact information in page footer). These kits are part of the Cellomics Total Solution Platform for HCS, which also includes Cellomics ArrayScan and other HCS Instrumentation, BioApplication Image Analysis Software and High-Content Informatics. For more information, visit www.thermo.com/cellomics or call 800-432-4091 (toll free) or 412-770-2500.

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