

Cellomics® Phospho-ATM Activation Kits

For High-Content Screening

1992.0

Number	Description
8403001	Phospho-ATM Activation Kit , sufficient materials for 1 × 96 wells
8403002	Phospho-ATM Activation Kit , sufficient materials for 5 × 96 wells

Kit Contents	8403001	8403002
Phospho-ATM Primary Antibody	12 µl	60 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-ATM 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-ATM Activation Kit contains optimized reagents for the detection and quantitation of phosphorylated ATM (Ser1981) in the nuclei. The kit contains a primary monoclonal antibody that detects only the phosphorylated form of human ATM, 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.

Ataxia telangiectasia mutated kinase (ATM, 350 kDa) is involved in cell cycle check-point signaling and DNA repair. Mutation in the ATM gene leads to ataxia telangiectasia, an autosomal recessive human disease. ATM is auto phosphorylated at Ser1981 upon induction of DNA double-strand breaks (DSBs) leading to rapid check-point signaling. ATM kinase has several identified targets including H2AX, BRCA1, NBS1, Chk1, Chk2 and p53.¹⁻²

Phospho-ATM in the nucleus of A549 cells treated with etoposide was assayed using the Cellomics Phospho-ATM Activation Kit, the Cellomics ArrayScan® HCS Reader³ (Figure 1) and the Compartmental Analysis BioApplication Software Module. Induction of DNA damage by etoposide leads to phosphorylation of ATM. Cells labeled using this kit also can be imaged by fluorescence or confocal microscopy.

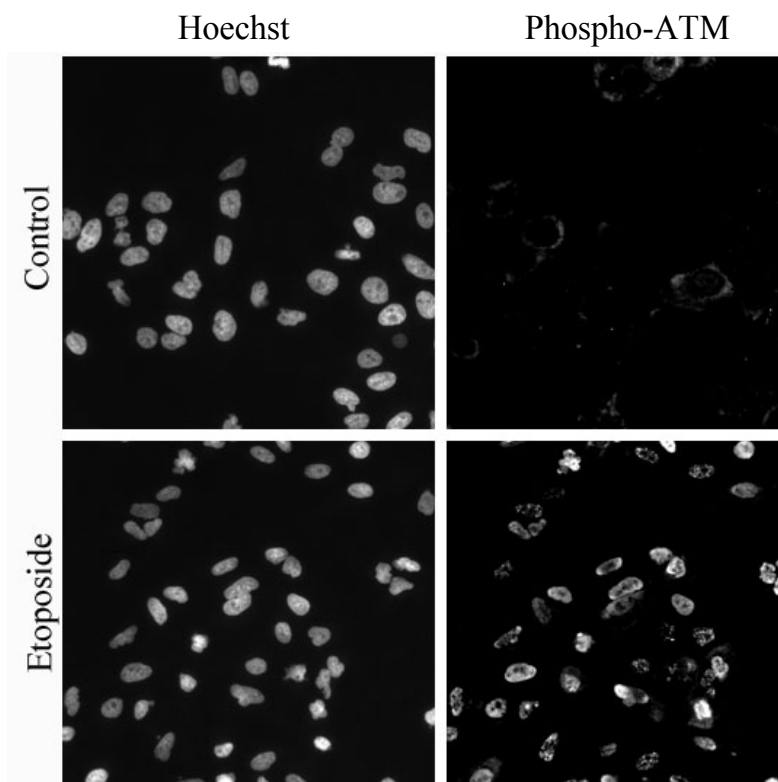


Figure 1. Staining of Phospho-ATM in A549 cells. Cells were treated with vehicle (0.1% DMSO in media) or with 50 μ M etoposide for 3 hours, stained according to the kit protocol, and imaged using a Cellomics ArrayScan HCS Reader.

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). Using cells other than A549 may require optimization. This kit has also effectively stained the following cell types: HeLa, HT1080 and HepG2.
- For routine culture of cells, use F12K medium supplemented with 10% fetal bovine serum, 100 units/ml penicillin, and 100 μ g/ml streptomycin (F12K complete medium).
- Split cells when they reach 90% confluence at a dilution of 1:3. Use cells at a passage number \leq 20.
- For phospho-ATM detection, harvest cells by trypsinization, dilute into F12K complete medium and determine cell density. Dilute cells to 15×10^4 cells/ml in F12K complete medium and add 100 μ l of the cell suspension per well of a 96-well microplate to achieve the recommended plating density of 15,000 cells/well.
- Incubate cells for overnight at 37°C in 5% CO₂ before drug treatment.

Phospho-ATM 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 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 to 9 ml of 1X Wash Buffer just before use.
1X Blocking Buffer	Add 5 ml of 10X Blocking Buffer to 45 ml Wash Buffer. Store buffer at 4°C for up to 7 days.
1X Permeabilization Buffer	Add 1.5 ml of 10X Permeabilization Buffer to 13.5 ml of 1X Wash Buffer. Store buffer at 4°C for up to 7 days.
Primary Antibody Solution	Add 12 µl of Anti-Phospho-ATM Primary Antibody to 6 ml of 1X Blocking Buffer. Prepare solution just before each assay.
Staining Solution	Add 0.6 µl of Hoechst Dye and 12.0 µl of the DyLight 549 Goat Anti-Mouse 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 3 hours 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 (RT) for 15 minutes.
3. Aspirate Fixation Solution completely, and then wash plate twice with 100 µl/well of 1X Wash Buffer.
4. Aspirate Wash Buffer completely, add 100 µl/well of 1X Permeabilization Buffer and incubate for 15 minutes at RT.
5. Aspirate Permeabilization Buffer and wash plate twice with 100 µl/well of 1X Wash Buffer.
6. Aspirate Wash Buffer, add 100 µl/well 1X Blocking Buffer supplemented with 2% fetal bovine serum (FBS), 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 RT.
8. Aspirate Primary Antibody Solution and wash with 100 µl/well of 1X Wash Buffer II.
9. Aspirate Wash Buffer II and wash twice with 100 µl/well of 1X Wash Buffer.
10. Aspirate Wash Buffer and add 50 µl/well of Staining Solution containing the goat anti-mouse secondary antibody. Incubate plate at RT for 45 minutes.
11. Aspirate Staining Solution and wash plate twice with 100 µl/well of 1X Wash Buffer II.
12. Aspirate Wash Buffer II and replace with 200 µl/well of 1X Wash Buffer.
13. Seal plate and evaluate on the ArrayScan HCS Reader or by fluorescence microscopy. Store plates at 4°C.

Additional Information

A. Dose Response Curve

A549 cells were treated with different doses of etoposide for 3 hours as described in the procedure, and intensity of phospho-ATM was measured (Figure 2). The feature plotted is the Mean_CircRingAvgIntDiff, which is the difference between nuclear and cytoplasmic intensities.

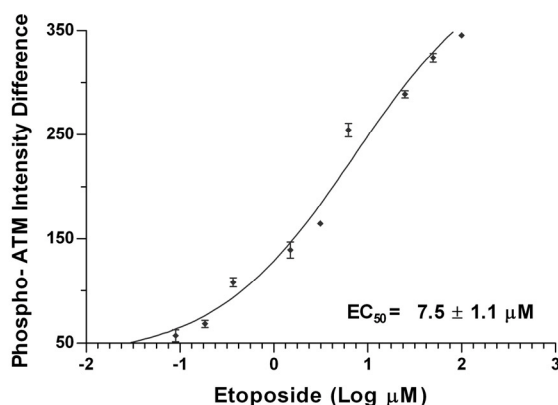


Figure 2. Dose response curve for etoposide-treated A549 cells. Etoposide concentration is plotted against the difference between nuclear and cytoplasmic intensities for phospho-ATM. Data represents mean \pm SD from three plates (eight wells per 96-well plate per dose of etoposide).

B. Performance Robustness

The robustness of the kit was ascertained by determining the Z' for the Mean_CircRingAvgIntDiff in non-treated (vehicle) and etoposide- (50 μM) treated cells.⁴ The Z' for phospho-ATM activation was 0.58 ± 0.01 .

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 CO₂ 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.

<u>Kit Component</u>	<u>96-Well Plates</u>	<u>384-Well Plates</u>	<u>24-Well Plates</u>
	<u>(μl/well)</u>	<u>(μl/well)</u>	<u>(μl/well)</u>
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

1. Kastan M.B. and Lim D.S. (2000). The many substrates and functions of ATM. *Nature Rev Mol Cell Biol* 1:179-86.
2. Lavin, M.F., *et al.* (2005) ATM signaling and genomic stability in response to DNA damage. *Mutat Res* 569:123-32.
3. 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.
4. 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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