

# INSTRUCTIONS

## Cellomics® Phospho-ATM and p53 Activation Kit

### High-Content Screening Reagents

2044.0

Number	Description
8405701	<b>Phospho-ATM and p53 Activation Kit</b> , materials for 1 × 96 wells
8405702	<b>Phospho-ATM and p53 Activation Kit</b> , materials for 5 × 96 wells

Kit Contents:	8405701	8405702
p53 Primary Antibody (rabbit)	24 µl	120 µl
Phospho-ATM Primary Antibody (mouse)	12µl	60 µl
DyLight™ 549 Conjugated Goat Anti-Rabbit IgG	14 µl	72 µl
DyLight 488 Conjugated Goat Anti-Mouse IgG	14 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 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 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. Kit is shipped with an ice pack.

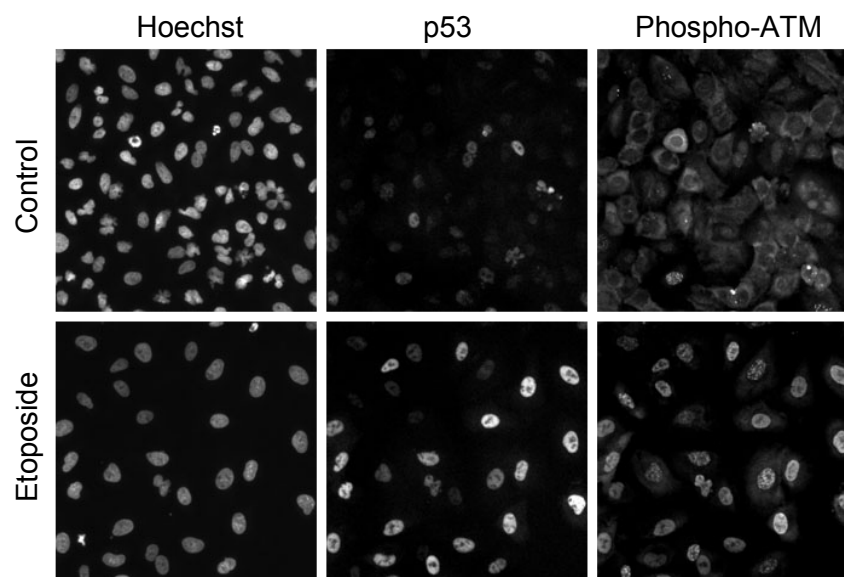
**Warning:** Please completely read these instructions and the accompanying material safety data sheets before using this product. Cellomics Reagents are not for diagnostic use in humans or animals.

### Introduction

The Cellomics Phospho-ATM and p53 Activation Kit contains optimized reagents for the detection and quantitation of phosphorylated ATM (Ser1981) and p53 in the nucleus. The kit contains a monoclonal antibody that detects only the phosphorylated form of human ATM, anti-p53 polyclonal antibody, DyLight-conjugated Secondary Antibodies and various other reagents and buffers for immunofluorescence staining for high-content screening (HCS) assays.

Ataxia telangiectasia mutated kinase (ATM) 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 (350 kDa) is auto phosphorylated at Ser1981 upon induction of DNA double-strand breaks (DSBs) leading to rapid cellular check-point signaling and cell-cycle arrest. The tumor suppressor protein p53 is phosphorylated at Ser15 by ATM in response to DNA damage. ATM and p53 are important in protecting genomic integrity.<sup>1-3</sup>

To measure phospho-ATM and p53 levels, A549 cells were treated with etoposide. Induction of DNA damage by etoposide leads to phosphorylation of ATM and an increase of p53 in the nucleus (Figure 1). The output parameter for this assay is the difference in nuclear and cytoplasmic intensity or average nuclear intensity for phospho-ATM and average nuclear intensity for p53. The assay was developed in A549 cells and optimized using the Thermo Scientific ArrayScan® HCS Reader<sup>4</sup> and Compartmental Analysis Bioapplication Software Module. Cells stained using this kit also can be imaged using fluorescence or confocal microscopy.



**Figure 1. Staining of p53 and phospho ATM.** A549 cells were treated with vehicle (0.1% DMSO in media) or etoposide (50  $\mu$ M) for 24 hours. Cells were stained according to the kit protocol and imaged using the ArrayScan HCS Reader.

### Additional Materials

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

### Cell Preparation Information

- This protocol is optimized for A549 cells (American Type Culture Collection # CCL-185). HepG2 cells have also been used successfully for this assay. Using cells other than A549 will require protocol optimization.
- For routine culture of cells, EMEM Complete Medium containing the following supplements: 10% fetal bovine serum, 100 units/ml penicillin and 100  $\mu$ g/ml streptomycin (EMEM Complete Medium).
- Split cells when they reach 90% confluence at a dilution of 1:3. Use cells at a passage number  $\leq$  20.
- For the detection of phospho-ATM and p53, harvest cells by trypsinization, dilute into EMEM Complete Medium, and determine cell density. Dilute cells to  $15 \times 10^4$  cells/ml in EMEM Complete Medium and add 100  $\mu$ l of the cell suspension per well of a 96-well microplate to achieve the recommended plating density of 10,000 cells/well.
- Incubate cells overnight at 37°C in 5% CO<sub>2</sub> before drug treatment.

## Phospho-ATM and p53 Activation Kit Protocol

### Solution Preparation (per 96-well plate)

1X Wash Buffer	Add 20 ml 10X Wash Buffer to 180 ml ultrapure water. Store buffer at 4°C for up to 7 days.
1X Wash Buffer II	Add 20 ml 10X Wash Buffer II to 180 ml ultrapure water. Store buffer at 4°C for up to 7 days.
Fixation Solution	Add 3 ml of 16% formaldehyde to 9 ml of 1X Wash Buffer just before use.
Primary Antibody Blocking Buffer	Add 2.5 ml of FBS to 47.5 ml of 1X Wash Buffer to make 50 ml of 1X Blocking Buffer. Prepare just before each assay.
1X Blocking Buffer	Add 10 ml of 10X Blocking Buffer to 90 ml Wash Buffer. Store buffer at 4°C for up to 7 days.
1X Blocking Buffer with FBS	Add 10 ml of 10X Blocking Buffer to 88.0 ml Wash Buffer. Supplement with 2 ml of FBS. Prepare just before each assay.
1X Permeabilization Buffer	Add 10 ml of 10X Permeabilization Buffer to 90 ml of ultrapure water. Store buffer at 4°C for up to 7 days.
Primary Antibody Solution	Add 24 µl of p53 Primary Antibody and 12 µl of Phospho-ATM Primary Antibody to 6 ml of 1X Blocking Buffer.
Secondary Antibody/Staining Solution	Add 0.6 µl of Hoechst Dye, 12 µl of DyLight 549 Goat Anti-Rabbit and 12 µl of the DyLight 488 Goat Anti-Mouse to 6.0 ml of 1X Blocking Buffer. Prepare solution just before each assay.

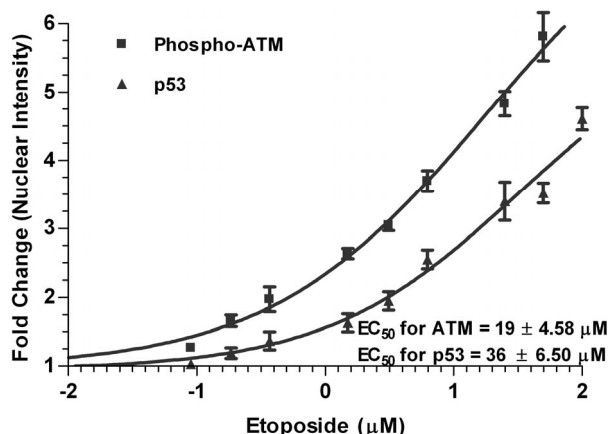
### Procedure

1. Prepare 2X solution of etoposide (100 µM) and add 100 µl to the cells. Incubate for 3 hours at 37°C.
2. Aspirate culture medium and add 100 µl/well of Fixation Solution. Incubate plate in a fume hood at room temperature for 15 minutes.
3. Aspirate Fixation solution and wash plate twice with 100 µl/well of 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 1X Blocking Buffer supplemented with 2% FBS and incubate at room temperature for 15 minutes.
7. Aspirate Blocking Buffer and add 50 µl/well of Primary Antibody Solution. Incubate for 1 hour at room temperature.
8. Aspirate Primary Antibody Solution and wash plate twice with 100 µl/well of 1X Wash Buffer II.
9. Aspirate Wash Buffer II and wash plate twice with 100 µl/well of 1X Wash Buffer.
10. Aspirate Wash Buffer and add 50 µl/well of Secondary Antibody/Staining Solution. Incubate for 45 minutes protected from light at room temperature.
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 Curves

Phospho-ATM and p53 were measured in A549 cells as described in the procedure in response to different doses of etoposide treatment for 24 hours (Figure 2). The feature plotted is the fold change of nuclear intensities for phospho-ATM and p53.



**Figure 2. Dose response curves for phospho-ATM and p53 treated with etoposide.** Data represents mean  $\pm$  SD from three plates (eight wells per 96-well plate per dose of etoposide).

### B. Performance Robustness

Assay robustness was ascertained by determining the  $Z'$  for the difference in nuclear and cytoplasmic intensities in non-treated cells (min, non-treated) and cells treated with 50  $\mu$ M for 24 hours (max). The mean  $\pm$  SD of the  $Z'$  factors were determined from three plates of A549 cells that were treated identically and were as follows:

phospho-ATM:  $0.46 \pm 0.11$  CV 11%

p53:  $0.44 \pm 0.03$  CV 13%

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 might be required when using slides, cover slips 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 (orange)

DyLight 488 Conjugates = 494/532 nm (green)

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 Thermo Scientific Multidrop<sup>®</sup> Combi or WellMate<sup>®</sup> Dispensers.
- **Dead Volumes:** Every piece of automation instrumentation has an associated non-recoverable dead volume. 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 might 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<sub>2</sub> 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>ul/well</u> )	<u>384-Well Plates</u> ( <u>ul/well</u> )	<u>24-Well Plates</u> ( <u>ul/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

S50-5011-1 or S50-2011-1

Target Detection BioApplication

S50-5017-1 or S50-2017-1

Compartmental Analysis BioApplication

## General 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. Efeyan, A. and Serrano, M. (2007). p53: a guardian of the genome and policeman of oncogenes. *Cell Cycle* **6(9)**:1006-10.
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](http://www.thermo.com/cellomics) or call 800-432-4091 (toll free) or 412-770-2500.

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