

Cellomics[®] Multiplex Mitosis-Apoptosis Kit

High-Content Screening Reagents

2096.0

Number	Description
8408202	Multiplex Mitosis-Apoptosis Kit , materials for 5 × 96 wells
8408203	Multiplex Mitosis-Apoptosis Kit , materials for 50 × 96 wells

Kit Contents:	8408202	8408203
BrdU Primary Antibody	600 µl	10 x 600 µl
Active Caspase 3 Primary Antibody	30 µl	10 x 30 µl
p53 Primary Antibody	30 µl	10 x 30 µl
DyLight™ 488 Conjugated Goat Anti-Mouse IgG	72 µl	1 ml
DyLight 549 Conjugated Goat Anti-Rabbit IgG	72 µl	1 ml
DyLight 649 Conjugated Donkey Anti-Goat IgG	72 µl	0.8 ml
BrdU	100 µl	6 x 100 µl
Magnesium Chloride (1 M)	500 µl	5 x 500 µl
DAPI Dye	50 µl	5 x 50 µl
Wash Buffer (10X Dulbecco's PBS)	100 ml	-
Permeabilization Buffer (10X Dulbecco's PBS with 1% Triton [®] X-100)	100 ml	-
Blocking Buffer (10X)	85 ml	-
Thin Plate Seal Assembly	7/pack	-

Storage: Store the BrdU Primary Antibody, Active Caspase 3 Primary Antibody and p53 Primary Antibody at -20°C. Store all other kit components at 4°C. Store the fluorescent antibodies and DAPI 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. Cellomics Reagents are not for diagnostic use in humans or animals.

Introduction

The Thermo Scientific Cellomics Multiplex Mitosis-Apoptosis Kit is for simultaneous quantification of nuclear DNA content, BrdU incorporation and active caspase 3 and p53 proteins (Figure 1). This kit allows simultaneous measurements of cell proliferation (cell number, DNA replication) and apoptosis using a fixed end-point assay based on immunofluorescence detection in cells grown on standard high-density microplates. DAPI is a DNA-binding dye used to determine the nuclear size and nuclear morphology as well as cell cycle phases by DNA content. The primary antibodies are specific for their targets and have minimal cross-reactivity.

This kit has been optimized with the Thermo Scientific ArrayScan HCS Reader using the Compartmental Analysis BioApplication Software Module. Thus, automated plate-handling, focusing, cell image acquisition/processing, and data analysis/management are combined in one high-content screening (HCS) system to assay for test compounds.¹ In addition to HCS instruments, cells labeled by the kit reagents can be viewed and analyzed by other fluorescence microscopes.

Cell proliferation and apoptosis, which denote life and death of the cell, are critical areas of cell biology and drug-discovery research.² This kit identifies cell proliferation (BrdU), caspase-dependent cell death pathway and p53 pathway in the cell, which enables systematic investigation of apoptosis and mitosis events (Figures 1-2).

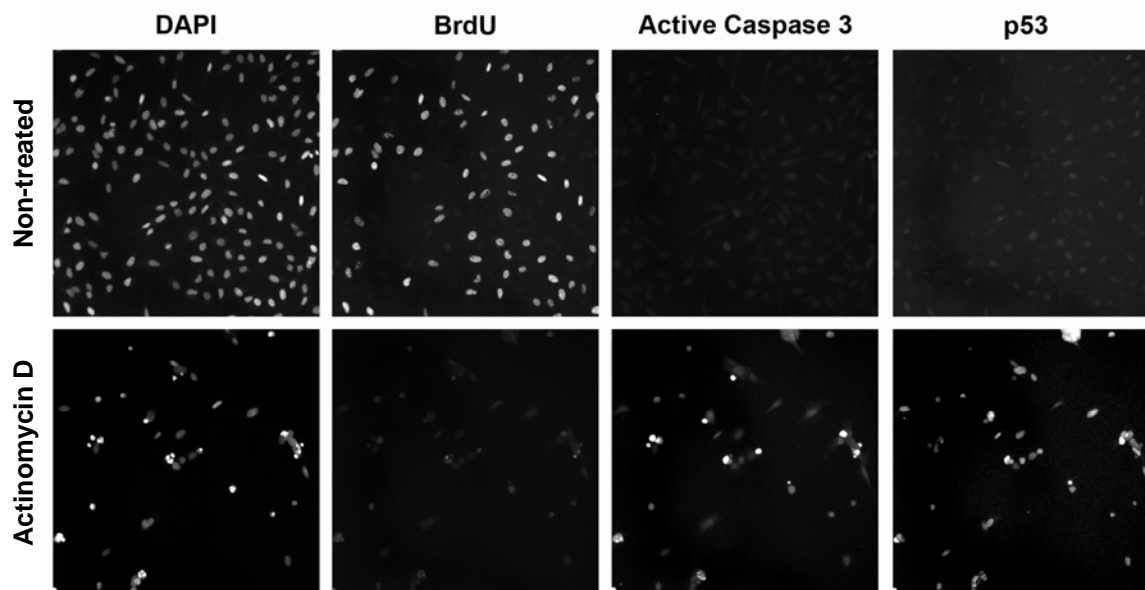


Figure 1. Staining of treated and non-treated A549 cells (10X objective). Markers were detected according to the kit protocol. The cells were treated with 1.6 μ M actinomycin D, pulse-labeled with BrdU for 30 minutes and probed with BrdU, active caspase 3 and p53 antibodies followed by corresponding DyLight Secondary Antibodies. Cell images were acquired using the ArrayScan HCS Reader.

Additional Material Required

Note: For the screening size kit, Wash Buffer, Permeabilization Buffer and Blocking Buffer are available separately. Please contact customer service for more information.

- Actinomycin D (Fisher Scientific Product No BP606-5) or other caspase 3 and p53 inducers
- Paraformaldehyde (16%) (Thermo Scientific Product No. 28908 or 28906)
- Collagen-I coated clear-bottom 96-well microplates (e.g., BD BioCoat™ Plate, Product No. 354407)

Cell Preparation Information

- This protocol is optimized for A549 lung carcinoma cells (American Type Culture Collection Product No. CCL-185). MDCK, IMR-90 and NIH 3T3 cell lines have been tested successfully for caspase 3 and p53. Using cell lines other than A549 might require protocol optimization.
- For routine culture of A549 cells use EMEM Complete Media (Thermo Scientific HyClone Product No. SH30024) supplemented with 10% fetal calf serum, 100 units/ml penicillin, 100 μ g/ml streptomycin, 1X non-essential amino acids and 1 mM sodium pyruvate.
- Split cells when they reach 70-80% confluence (2-3 times per week) at a ratio of 1:3-1:8. Use cells at a passage number \leq 20.
- Harvest cells by trypsinization, dilute into EMEM Complete Medium and determine cell density. Dilute cells to 10^5 cells/ml in EMEM Complete Medium and add 100 μ l of the cell suspension per well of a 96-well microplate (recommended plating density = 10,000 cells/well).

Multiplex Mitosis-Apoptosis Kit Protocol

- The total intensity from a DAPI-labeled nucleus, determined on an image analysis system such as on ArrayScan HCS Reader, is proportional to the nuclear DNA content. The linear range of the dye can vary depending on cell type. Incubating BrdU primary antibody longer than 1 hour at 37°C may diminish DAPI staining intensity.
- Centrifuge the dye and the antibody vials briefly before use.

A. 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.
Formaldehyde (16%)	Immediately before use, warm 10 ml of the 16% formaldehyde to 37°C.
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 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 60 µl of MgCl ₂ , 110 µl of BrdU Primary Antibody, 6 µl of Active Caspase 3 Primary Antibody and 6 µl of p53 Primary Antibody to 6.0 ml of 1X Blocking Buffer. Prepare solution just before each assay.
Staining Solution 1	Add 12 µl of the DyLight 649 Donkey Anti-Goat to 6.0 ml of 1X Blocking Buffer.
Staining Solution 2	Add 3 µl of DAPI, 12 µl of DyLight 488 Goat Anti-Mouse and 12 µl of the DyLight 549 Goat Anti-Rabbit to 6.0 ml of 1X Blocking Buffer. Prepare solution just before each assay.

B. Procedure (with a 96 well plate)

1. Plate 10,000 cells of A549 in 100 µl EMEM complete media per well and incubate 16-24 hours at 37°C in 5% CO₂. Assign control wells in the microplate.
2. Prepare 1.6 mM actinomycin D stock solution in sterile DMSO. Dilute actinomycin D to 4.8 µM in culture medium (e.g., 30 µl of 1.6 mM actinomycin D in 10 ml EMEM complete media) or other test compound to appropriate concentration. Add 50 µl of 4.8 µM actinomycin D to each treatment well and add 50 µl of culture medium to the control wells. Incubate plate for 24 hours at 37°C in 5% CO₂.
3. Dilute BrdU (100 mM) to 160 µM in warm (37°C) culture medium (e.g., 12 µl of BrdU in 7.5 ml of medium per plate). Add 50 µl of BrdU in culture medium to each control and treatment well and incubate for 30 minutes at 37°C in 5% CO₂.
4. Add 60 µl/well of warm 16% formaldehyde and incubate in a fume hood at room temperature (RT) for 30 minutes.
5. Aspirate formaldehyde and wash plate twice with 100 µl/well of 1X Wash Buffer.
6. Aspirate buffer, add 100 µl/well of 1X Permeabilization Buffer and incubate for 15 minutes at RT.
7. Aspirate Permeabilization Buffer, add 100 µl/well of 1X Blocking Buffer and incubate for 15 minutes at RT.
8. Aspirate Blocking Buffer and add 50 µl/well of Primary Antibody Solution. Incubate plate for 1 hour at 37°C.
Note: To reduce variation between wells when using multiple plates, spread plates in the incubator (i.e., do not stack plates). Room temperature incubation will diminish signal intensity.
9. Aspirate Primary Antibody Solution and wash plate twice with 100 µl/well of 1X Blocking Buffer.
10. To avoid cross-reactivity between the secondary antibodies, sequentially add antibodies. Aspirate Blocking Buffer and add 50 µl/well of Staining Solution 1 containing DyLight 649 Donkey Anti-Goat secondary antibody. Incubate for 30 minutes protected from light at RT.
11. Aspirate Staining Solution and wash plate twice with 100 µl/well of 1X Blocking Buffer.
12. Aspirate buffer and add 50 µl/well of Staining Solution 2. Incubate plate for 30 minutes at RT protected from light.
13. Aspirate Staining Solution and wash plate twice with 100 µl/well of 1X Wash Buffer. Aspirate buffer and add 100 µl/well of 1X Wash Buffer.
14. Seal plate and evaluate on the ArrayScan HCS Reader. Store plates at 4°C.

Additional Information

A. Application Data

Actinomycin D and camptothecin (treated for 24 hours) increase caspase 3 and p53 intensity but decrease BrdU intensity in the nucleus of A549 cells (Figure 2).

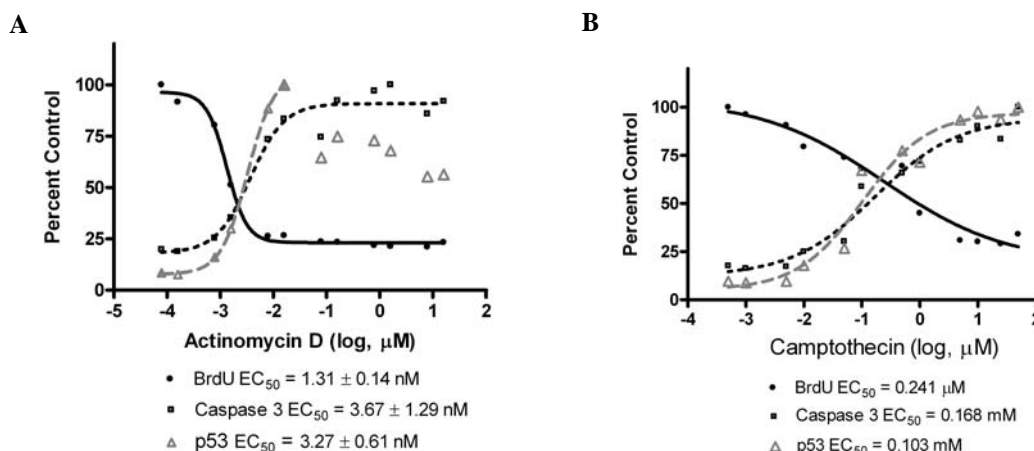


Figure 2. Drugs induce Caspase 3 and p53 but inhibit cell proliferation in A549 cells. Different drugs were added to the cells at the indicated concentrations. Each data point represents eight wells from three plates (Panel A). BrdU, caspase 3 and p53 were measured using the output parameter of nuclear fluorescence intensity with Cellomics Compartmental Analysis Bioapplication. Values are normalized with the maximum control value and presented as percent control.

B. Performance Robustness

The robustness of the kit was ascertained by determining the Z' factor for the average intensity of the nuclear fluorescence in non-treated cells (min, 0 μM) and cells treated with actinomycin D (1.6 μM) for 24 hours.³ The Z' were determined from three plates of A549 cells that were treated identically. The Z' factors were 0.30 ± 0.02 , with a maximum CV of 10.1% (BrdU); 0.45 ± 0.05 , with a maximum CV of 12.3% (active caspase 3); and 0.74 ± 0.02 , with a maximum CV of 7.9% (p53).

Note: for BrdU detection, population analysis (e.g., percent response) may give robust performance in other cell types.

- DMSO tolerance: Assay performance using these kits was robust when compounds were added in DMSO at $\leq 2\%$.

C. Microscope Information

Cells prepared and labeled according to this kit protocol 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 488 Conjugates = 494/532 nm

DyLight 549 Conjugates = 550/568 nm

DyLight 649 Conjugates = 646/674 nm

DAPI Dye = 358/461 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 pipeting 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>µl/well</u>)	<u>384-Well Plates</u> (<u>µl/well</u>)	<u>24-Well Plates</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

S50-0017-2, S50-5017-1 or S50-2017-1 **Compartmental Analysis BioApplication**

S50-0016-1 or S50-5016-1 or S50-2016-1 **Target Activation BioApplication**

S50-0001-2 **Cytoplasm to Nucleus Translocation BioApplication**

S50-0019-2 **Molecular Translocation BioApplication**

References

1. 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.
2. Roberts, R.A., *et al.* (2007) Perturbation of the mitosis/apoptosis balance: A fundamental mechanism of toxicology. *Fundam. Appl. Toxicol.* **38**:107-15
3. 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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