

INSTRUCTIONS

Cellomics[®] HCS Reagent Kits Caspase 9 Activation Kits

For High Content Screening

1966.1

Number	Description
8402301	Caspase 9 Activation Kit, sufficient materials for 1 × 96 wells
8402302	Caspase 9 Activation Kit, sufficient materials for 5 × 96 wells

Kit Contents:	8402301	8402302
Cleaved Caspase 9 Primary Antibody	15 µl	75 µl
DyLight™ 549-Conjugated Goat Anti-Rabbit IgG	19 µl	70 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
Goat Serum	2 ml	2 ml
Thin Plate Seal Assembly	7/pack	7/pack

Storage: Store kit 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. Store the Cleaved Caspase 9 Primary Antibody at -20°C. 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 diagnostics in humans or animals.

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Introduction

The Cellomics Caspase 9 Activation Reagent Kits contain optimized reagents for the detection and quantitation of caspase 9 activation in cells (Figure 1). These kits allow direct in-cell measurements using a fixed end-point assay based on immunofluorescence detection in cells grown on standard high-density microplates. The primary antibody is specific for human cleaved caspase 9 and does not cross-react with total caspase 9 or other caspases. The secondary antibody is conjugated to DyLight 549 Fluor (orange).

The kit reagents have been optimized for use with the ArrayScan[®] HCS Reader and the Target Activation BioApplication Software Module, but they also can be used with other Cellomics BioApplication Modules (see the Compatible BioApplication Software Modules Section). Thus, automated plate-handling, focusing, cell image acquisition/processing, and data analysis and management have been combined into a high-content screening (HCS) system to assay for test compounds affecting the activation of caspase 9. In addition to HCS instruments, cells labeled by the reagents in this kit can be viewed and analyzed by other fluorescence microscopes.

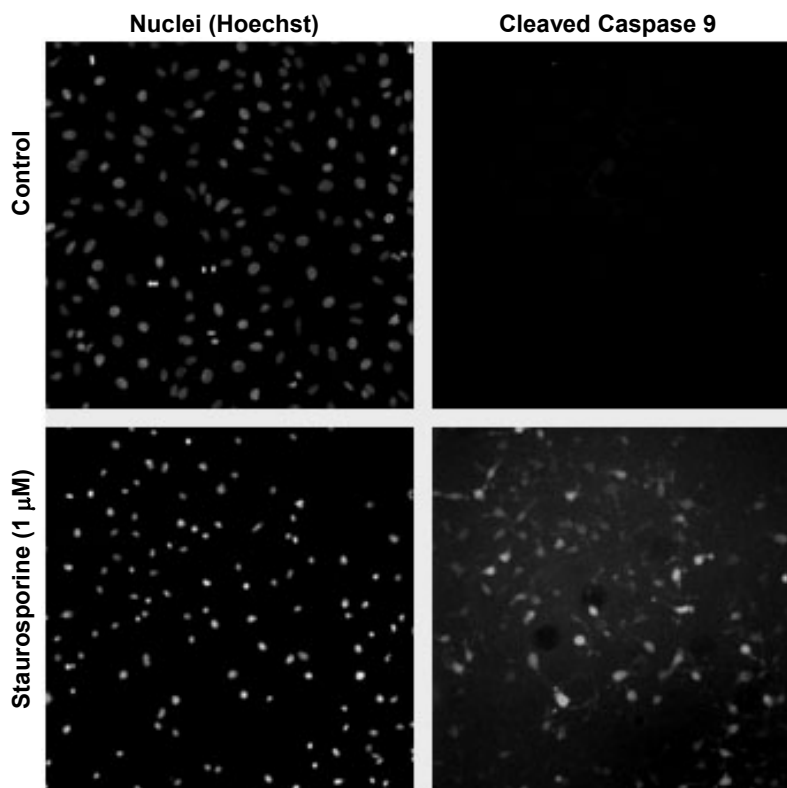


Figure 1. Staining of cleaved (active) caspase 9 in HeLa cells treated with vehicle (0.1% DMSO in media) or with 1 μ M staurosporine for 4 hours. Cells were stained according to the kit protocol and imaged using a Cellomics ArrayScan HCS Reader. Cells treated with staurosporine results in caspase 9 activation and an increase in staining.

Background

Caspases are intracellular cysteine proteases that are important in apoptotic cell death in a variety of cell lines. Caspase 9 (ICE-LAP6, Mch6) is a member of mitochondrial pathway of apoptosis. Apoptotic induction by this pathway leads to release of cytochrome c from mitochondria that associates with pro-caspase 9 and Apaf1 leading to self cleavage of pro-caspase 9 at Asp315 and activation. The proteolytic processing of pro-caspase 9 is regulated by Akt phosphorylation at Ser196. Activated caspase 9 cleaves and activates caspase 3 leading to DNA fragmentation and apoptosis.¹⁻³ Caspase 9 is identical to Apaf-3. The Cellomics Caspase 9 Activation Reagent Kit along with the ArrayScan HCS Reader and the Target Activation BioApplication Software Module enable the quantitation of active caspase 9 in cells.

Additional Material Required

- Ultrapure water
- Paraformaldehyde (16%) (Fisher Scientific Inc., # NC9368642)
- Packard View 96-well microplates (Perkin-Elmer, # 6005182)
- Positive control compound (e.g., staurosporine, Sigma, # S3921)

Procedure for Preparing the Cells

- This protocol is optimized for HeLa cells (American Type Culture Collection #CCL-2) cultured in 96-well plates. Using conditions other than those indicated may necessitate optimization. This kit is also effective on A549 and HepG2 cells. Please see the website for the latest updated list on compatible cell types.
- To culture cells, use MEM medium containing 10% fetal bovine serum, 1 mM sodium pyruvate, non-essential amino acids and 100 units/ml penicillin, and 100 µg/ml streptomycin (= MEM complete medium).
- Split cells when they reach 90% confluence at a dilution of 1:3. Use cells at a passage number ≤ 20.
- Harvest cells by trypsinization, dilute into MEM complete medium and determine cell density. Dilute cells to a density of 10⁵ cells/ml in MEM complete medium and add 100 µl of the cell suspension per well of a 96-well microplate to achieve 10,000 cells/well.
- Incubate cells overnight at 37°C in 5% CO₂ before treatment.

Notes for the Active Caspase 9 Assay Protocol

- Do not allow plate wells to become dry at any time during the protocol.
- Perform all steps at room temperature unless otherwise indicated.
- Make compound solutions immediately before use.
- The protocol requires approximately 3 hours post-compound treatment to complete.
- Please refer to the Compatible BioApplication Software Modules Section for Cellomics applications that can be used with this kit and the ArrayScan HCS Reader instructions for optimal assay implementation.
- DyLight 549 Conjugates have an approximate absorption/emission maxima of 562/572 nm. Hoechst Dye has an approximate absorption/emission maxima of 350/461 nm.
- The total intensity from a Hoechst-labeled nucleus, determined on an image analysis system such as the ArrayScan HCS Reader, is proportional to the nucleus DNA content. Hoechst staining can be used to determine cell-cycle phase within the linear range of the dye only. The dye's linear range can vary depending on cell type.
- DMSO tolerance: Assay performance using these kits was robust when compounds were added with up to 1% DMSO.
- Cells prepared and labeled according to the instructions in this kit can be used and analyzed by fluorescent microscopy-based instrumentation 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.

Active Caspase 9 Assay Kit Protocol

A. Solution Preparation (per 96-well plate)

1X Wash Buffer	Add 20 ml of 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 of 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. Store this buffer at 4°C for up to 7 days. Supplement the blocking buffer with 1% normal goat serum.
Goat Serum	Add 2 ml of ultrapure water to the vial containing lyophilized goat serum
Primary Antibody Solution	Add 15 µl of the Cleaved Caspase 9 Primary Antibody to 6 ml of 1X Blocking Buffer. Prepare solution just before each assay.
Secondary Antibody Solution	Add 0.6 µl of Hoechst Dye and 12 µl of each of the secondary antibodies (DyLight 549 Goat anti-Rabbit) to 6 ml of 1X Blocking Buffer. Prepare solution just before each assay.

B. Procedure

1. Prepare 2X solution of staurosporine (2 µM), add 100 µl to the cells and incubate for 4 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, 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 1X Wash Buffer.
6. Aspirate Wash Buffer, add 100 µl/well of 1X Blocking Buffer supplemented with 1% goat serum 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 buffer and add 50 µl/well of Secondary Antibody Solution. Incubate for 30 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 buffer 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

The caspase 9 activation reagent kits can be used to calculate reliable EC_{50} values using different doses of test compound. We measured caspase 9 activation in response to different doses of staurosporine (Figure 2). The percentage of cells that were responders was measured using the Target Activation BioApplication. EC_{50} values are indicated.

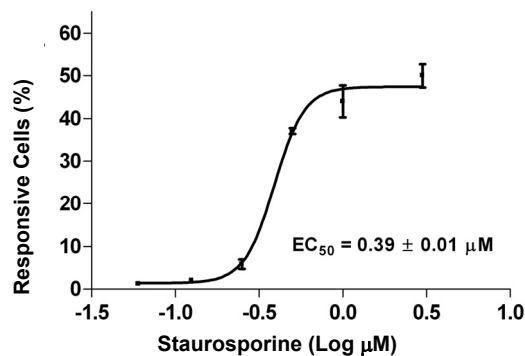


Figure 2. Caspase 9 activation in HeLa cells. The assay was performed as described in the procedure using different doses of staurosporine treatment for 4 hours. The feature plotted is the percent of cells that are responders for caspase 9. Data represents mean \pm SD from three plates (eight wells per 96-well plate per dose of staurosporine).

B. Performance Robustness of the Active Caspase 9 kits

Kit robustness was ascertained by determining the Z' for the percent average intensity responders in non-treated (vehicle) and staurosporine- (1 μM) treated wells. A positive Z' value means that an assay can be used for screening and a Z' of ≥ 0.5 means that this is an excellent assay for screening.⁴ The Z' of Active Caspase 9 Kit (1 μM staurosporine) is 0.63 ± 0.04 .

C. Recommendations for Automation

- **Plating Cells:** To improve the uniformity and throughput of plating cells, use a liquid handling system such as Titertek® Multidrop Dispenser.
- **Dead Volumes:** Every piece of automation instrumentation has a non-recoverable dead volume associated with it. Be aware of dead volumes, priming volumes and rinsing volumes when calculating 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 (μ l/well)	384-Well Plates (μ l/well)	24-Well Plates (μ l/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

S50-0011-1 or S50-2011-1

Target Detection BioApplication

S50-0017-2

Compartmental Analysis BioApplication

References

1. Kuida, K., *et al.* (1998). Reduced apoptosis and cytochrome c-mediated caspase activation in mice lacking caspase 9. *Cell* **94**(3):325-37.
2. Li, P., *et al.* (1998). Cytochrome c and dATP-dependent formation Apaf-1/caspase-9 complex initiates an apoptotic protease cascade. *Cell* **91**(4):479-89.
3. Zou, H., *et al.* (1997). An APAF-1 cytochrome c multimeric complex is a functional apoptosome that activates procaspase-9. *J Biol Chem* **274**(17):11549-56.
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.

The listed BioApplications Software Modules are protected by U.S. patent #5,989,835 and other patent pending.

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