

Cellomics[®] Caspase 3 Activation Kit

High-Content Screening Reagents

1965.1

Number	Description
8402201	Caspase 3 Activation Kit , sufficient materials for 1 × 96 wells
8402202	Caspase 3 Activation Kit , sufficient materials for 5 × 96 wells

Kit Contents:	8402201	8402202
Active Caspase 3 Primary Antibody	8 µl	30 µl
DyLight™ 549-Conjugated Goat Anti-Rabbit 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 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: 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 Active Caspase 3 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.

Introduction

The Thermo Scientific Cellomics Caspase 3 Activation Kits contain optimized reagents for detecting and quantitating caspase 3 activation (cleaved) 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 active caspase 3 from human, mouse and rat and does not recognize full-length caspase 3 or other caspases. The secondary antibody is conjugated to DyLight 549 Fluor (orange).

The kit reagents are optimized for use with the Thermo Scientific ArrayScan[®] HCS Reader and the Target Activation BioApplication Software Module, but they also can be used with other 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 3. In addition to HCS instruments, cells labeled by the reagents in this kit can be viewed and analyzed by other fluorescence microscopes.

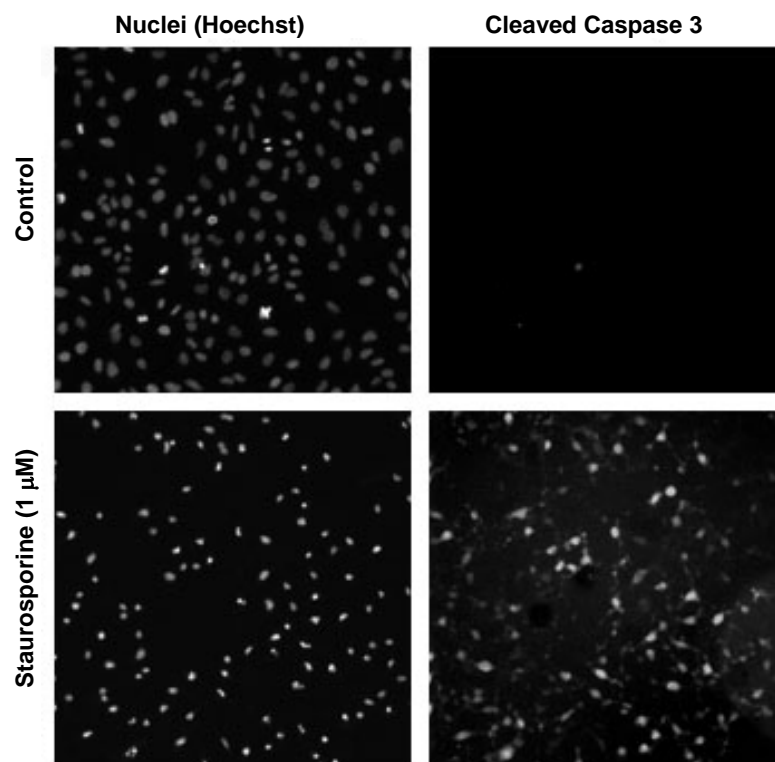


Figure 1. Staining of cleaved (active) caspase 3 in HeLa cells treated with vehicle (0.1% DMSO in media) or with 1 μM staurosporine for 4 hours. Cells were stained according to the kit protocol and imaged using a ArrayScan HCS Reader. Cells treated with staurosporine results in caspase 3 activation and an increase in staining.

Background

Caspases are intracellular cysteine proteases that are important in apoptotic cell death in a variety of cell lines. Caspase 3 can be activated by two different pathways: mitochondrial apoptosis and Fas ligand-mediated apoptosis. Caspase 3 exists as an inactive zymogen in cells and is activated by cleavage at Asp175 site by upstream caspases, caspase 9 in mitochondrial pathway and caspase 8 in Fas ligand-mediated apoptotic pathway.¹⁻³ Activated caspase 3 cleaves several substrates including PARP, leading to DNA fragmentation during apoptosis. Caspase 3 is also known as YAMA, apopain or CPP32. The active caspase 3 along with the ArrayScan HCS Reader and the Target Activation BioApplication Software Module enable the quantitation of active caspase 3 in cells.

Additional Material Required

- Ultrapure water
- Paraformaldehyde (16%) (Thermo Scientific 16% Formaldehyde, Product No. 28906)
- Packard View 96-well microplates (Perkin-Elmer, Product No. 6005182)
- Positive control compound, Staurosporine (Sigma, Product No. S3921)

Procedure for Preparing the Cells

- This protocol is optimized for HeLa cells (American Type Culture Collection, Product No. 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 an updated list on compatible cell types.
- Culture cells using 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 with MEM complete medium and determine cell density. Dilute cells to 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 3 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 fresh immediately before use.
- The protocol requires approximately 3 hours post-compound treatment to complete.
- Please refer to the Compatible BioApplication Software Modules Section for 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 these instructions can be 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.

Active Caspase 3 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. If desired, supplement the blocking buffer with 1% fetal bovine serum (FBS).
Primary Antibody Solution	Add 6 µl of Active Caspase 3 Primary Antibody to 6 ml of 1X Blocking Buffer without FBS. Prepare solution just before each assay.
Secondary Antibody/Staining Solution	Add 0.6 µl of Hoechst Dye and 12 µl of DyLight 549 Goat Anti-Rabbit to 6 ml of 1X Blocking Buffer without FBS. Prepare solution just before each assay.

B. Procedure

1. Prepare 2X solution of staurosporine (2 µM) and add 100 µl to the cells. Incubate cells for 4 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 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. Incubate plate 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 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/Staining Solution. Incubate plate for 30 minutes protected from light at room temperature.
11. Aspirate buffer and wash plate twice with 100 µl/well of 1X Wash Buffer.
12. Aspirate Wash Buffer and replace with 200 µl/well of 1X Wash Buffer.
13. Seal plate and evaluate on the ArrayScan HCS Reader. Store plates at 4°C.

Additional Information

A. Dose Response Curves

The caspase 3 activation reagent kits can be used to calculate reliable EC₅₀ values using different doses of test compound. We measured caspase 3 activation in response to different doses of staurosporine (Figure 2). The percentage of cells that were responders was measured using the Target Activation BioApplication. EC₅₀ values are indicated.

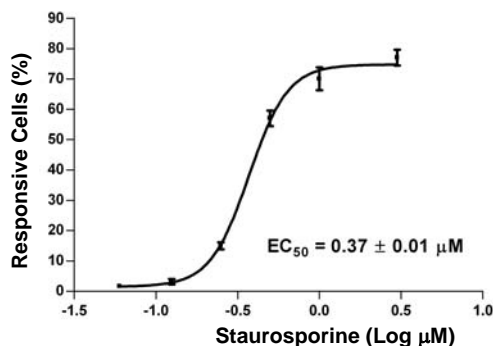


Figure 2. Caspase 3 activation in HeLa cells. The assay was performed as described in the procedure using different doses of staurosporine treatment for 4 hours. The feature plotted is the percent of cells that are responders for caspase 3. Data represents mean \pm SD from three plates (eight wells per 96-well plate per dose of staurosporine).

B. Performance Robustness

Kit robustness was ascertained by determining the Z' for the percent average intensity responders in non-treated (vehicle) and staurosporine (1 μ M treated wells). A positive Z' value means that an assay can be used for screening and a Z' \geq 0.3 means that this is an excellent assay for screening.⁴ The Z' of Active Caspase 3 Kit (1 μ M staurosporine) is 0.73.

C. Recommendations for Automation

- **Plating Cells:** To improve the uniformity and throughput of plating cells, use a liquid handling system such as 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 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 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.

Kit Component	96-Well Plates	384-Well Plates	24-Well Plates
	(μ l/well)	(μ l/well)	(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.* (1996). Decreased apoptosis in the brain and premature lethality in CPP32-deficient mice. *Cell* **384(6607)**:368-72.
2. Rosen, A. and Casciola-Rosen, L. (1997). Macromolecular substrates for the ICE-like proteases during apoptosis. *J Cell Biochem* **64(1)**:50-4.
3. Lakhani S.A., *et al.* (2006). Caspases 3 and 7: Key mediators of mitochondrial events of apoptosis. *Science* **311(5762)**:785-6.
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. Triton[®] is a registered trademark of Rohm & Haas.

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