

Cellomics[®] Cytochrome C Detection kit

High-Content Screening Reagents

2035.0

Number	Description
8405601	Cytochrome C Detection Kit, sufficient materials for 1 × 96 wells
8405602	Cytochrome C Detection Kit, sufficient materials for 5 × 96 wells

Kit Contents	8405601	8405602
Cytochrome C Primary Antibody	15 µl	75 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 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 Cytochrome C 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. The Cellomics Reagents are not for diagnostic use in humans or animals.

Introduction

The Cellomics Cytochrome C Detection Kit measures release of cytochrome c from mitochondria, a key early step in apoptosis or programmed cell death. The kit contains a mouse monoclonal anti-cytochrome c primary antibody, a secondary antibody conjugated to DyLight 549 Fluorophore, and various other reagents and buffers required for immunofluorescent detection of cytochrome c for high-content screening (HCS) assays.

Cytochrome c is a small (12 kDa) heme protein present on the inner mitochondrial membrane and is critical for oxidative phosphorylation and production of ATP in cells and the mitochondrial pathway of apoptosis. Upon apoptosis stimulation, cytochrome c is released from mitochondria and then associates with caspase 9/Apaf-1 to form a complex. This complex formation leads to caspase 9 and caspase 3 activation and nuclear fragmentation.^{1,2}

To measure cytochrome c levels, HeLa cells were treated with staurosporine (Figure 1). In normal cells cytochrome c is in the mitochondria, which can be detected as cytoplasmic spots. Upon apoptosis induction, cytochrome c is released from the mitochondria and diffuses into the nucleus. The assay was developed and optimized using the Thermo Scientific ArrayScan[®] HCS Reader and Compartmental Analysis Bioapplication Software Module.³ Stained cells also can be imaged using fluorescence or confocal microscopy.

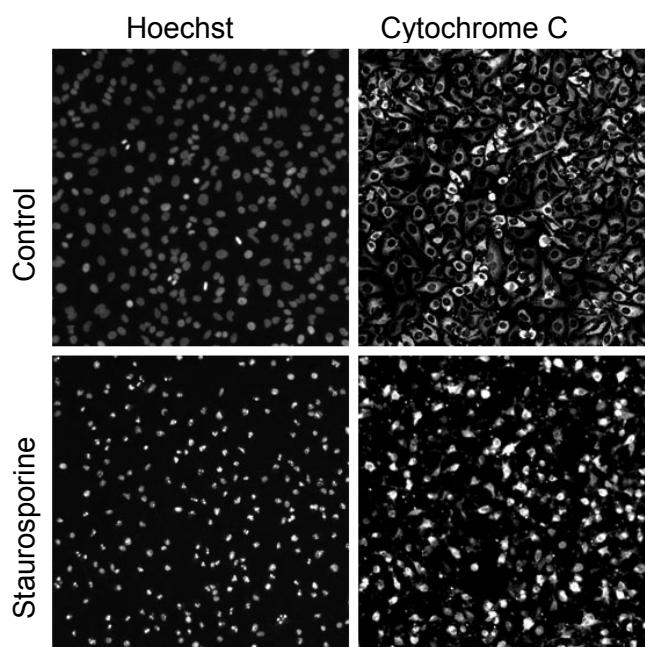


Figure 1. Staining of cytochrome c in HeLa cells treated with or without staurosporine (1 μ M) for 24 hours. Cells were stained according to the kit protocol and imaged using the ArrayScan HCS Reader. Staurosporine treatment leads to cytochrome c release from mitochondria and increased nuclear staining in treated cells.

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 staurosporine (Sigma Aldrich, Product No. S3921)
- Fetal bovine serum (FBS)

Cell Preparation Information

- This protocol is optimized for HeLa cells (American Type Culture Collection #CCL-2). HepG2 and A549 cells also have been used successfully in this assay. Using cells other than HeLa will require protocol optimization.
- For routine culture of cells, use EMEM Medium containing the following supplements: 10% fetal bovine serum, 1 mM sodium pyruvate, non-essential amino acids, 100 units/ml penicillin and 100 μ 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 ≤ 20 .
- For cytochrome c detection, 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 to achieve the recommended plating density of 10,000 cells/well.
- Incubate cells overnight at 37°C in 5% CO₂ before drug treatment.

Cytochrome C Detection 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 6 ml of 10X Wash Buffer II to 54 ml ultrapure water. 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 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 44 ml of 1X Wash Buffer and 1 ml of FBS for a final volume of 50 ml. Store this buffer at 4°C for up to 7 days.
Primary Antibody Solution	Add 15 µl of 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 the DyLight 549 Goat Anti-Mouse Antibody to 6 ml of 1X Blocking Buffer without FBS. Prepare solution just before each assay.

Procedure

1. Prepare 2X solution of staurosporine (2 µM) and add 100 µl to the cells and incubate for 4 hours at 37°C.
2. Add 65 µl/well of 16% formaldehyde, and incubate plate in a fume hood at room temperature for 15 minutes.
3. Aspirate formaldehyde 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 once 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

The cytochrome c in HeLa cells was measured in response to different doses of staurosporine treatment for 4 hours (Figure 2). The intensity difference between the nucleus and cytoplasm was used for quantitation.

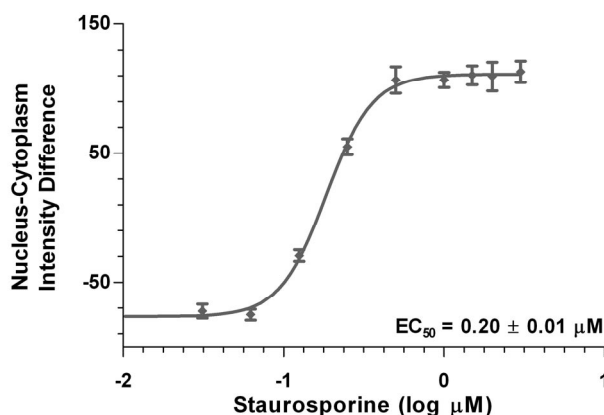


Figure 2. Dose response curve of cytochrome c in HeLa cells treated with staurosporine. Data represents mean \pm SD from three plates (eight wells per 96-well plate per dose of staurosporine).

B. Performance Robustness

Assay robustness was ascertained by determining the Z' factor for the average intensity difference between nucleus and cytoplasm of cytochrome c in nontreated- (vehicle) and staurosporine- ($1 \mu\text{M}$ for 4 hours) treated wells.⁴ The Z' was calculated using three plates of HeLa cells treated identically and was 0.56 ± 0.03 .

DMSO tolerance: The assay performance was robust when compounds were added with up to 1% DMSO.

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 may 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

Hoechst Dye = 350/461 nm

D. Automation Recommendations

- **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 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 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.

<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

S50-5001-1 or S50-2001-1	Cytoplasm to Nucleus Translocation BioApplication
S50-5019-1 or S50-2019-1	Molecular Translocation BioApplication
S50-5017-1 or S50-2017-1	Compartmental Analysis BioApplication

References

1. Li, P., *et al.* (1997). Cytochrome c and dATP-dependent formation of Apaf-1/caspase-9 complex initiates and apoptotic protease cascade. *Cell* **91(4)**:479-89.
2. Liu, X., *et al.* (1996). Induction of apoptotic program in cell-free extracts: requirement for dATP and cytochrome c. *Cell* **86(1)**:147-57.
3. Taylor, D.L., *et al.* (2007). High content screening: A powerful approach to systems cell biology and drug discovery. *Methods 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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