

Cellomics[®] Multiparameter Cytotoxicity 3 Kit

High-Content Screening Reagents

2069.2

Number	Description
8408102	Multiparameter Cytotoxicity 3 Kit, sufficient materials for 5 × 96 wells

Kit Contents	8408102
Cytochrome c Primary Antibody	75 µl
DyLight™ 649 Conjugated Goat Anti-Mouse IgG	72 µl
Mitochondrial Membrane Potential Dye	1 ea
Permeability Dye	25 µl
Hoechst Dye	30 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: Upon receipt immediately store the Cytochrome c Primary Antibody, Mitochondrial Membrane Potential Dye and Cell Permeability dye 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.

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

Introduction

The Thermo Scientific Cellomics Multiparameter Cytotoxicity 3 Kit enables simultaneous measurement of six orthogonal cell-health parameters: cell loss, nuclear morphology, DNA content, cell membrane permeability, mitochondrial membrane potential changes and cytochrome c localization and release from mitochondria (Figure 1). The kit contains a Hoechst dye, cell permeability dye, mitochondrial membrane potential dye, and a mouse monoclonal antibody against cytochrome c and a goat anti-mouse DyLight 649-conjugated Secondary Antibody, and various other essential reagents and buffers.

Screening potential drugs for toxicity is an essential aspect of the drug discovery process. *In vitro* toxicity assessments performed early in drug discovery are cost-effective and fast. Cytotoxicity is a complex process affecting multiple parameters and pathways. After toxic insult, cells often undergo either apoptosis or necrosis accompanied by changes in nuclear morphology, cell permeability and mitochondrial function, resulting in loss of mitochondrial membrane potential and release of cytochrome c from mitochondria.¹⁻⁶ A goal of *in vitro* toxicity testing is to detect the lowest dose of a compound that causes toxicity. Because of diverse action mechanisms with different compounds, monitoring multiple, independent toxicity indicators in the same cell increases the predictive power of the assay.

Cell-based high-content screening (HCS) assays enable quantitative measurements of multiple parameters related to cytotoxicity. This kit enables simultaneous measurements in the same cell of six independent parameters that monitor cell health, including cell loss, nuclear size and morphological changes, mitochondrial membrane potential changes, cytochrome c release, and changes in cell permeability (Figure 1). The Hoechst dye enables monitoring of cell loss, nuclear morphology changes and DNA content, which is proportional to the total Hoechst intensity per nucleus. The other three parameters are monitored by separate dyes.

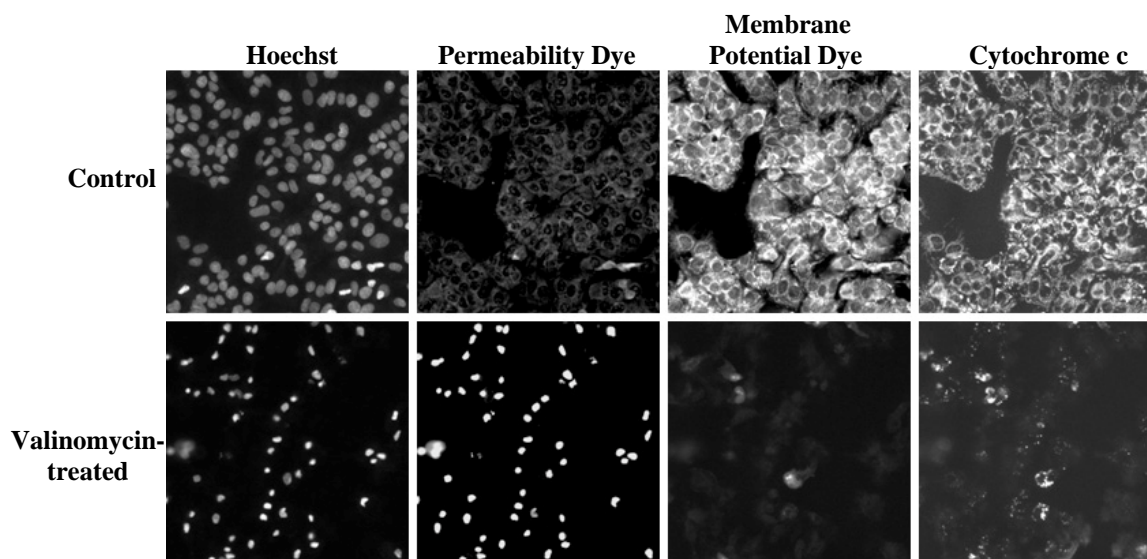


Figure 1. Staining of HepG2 cells. Cell loss, changes in nuclear size and morphology, DNA content, in mitochondrial membrane potential and cell permeability changes, and cytochrome c release were measured simultaneously. Cells were plated in 96-well collagen I-coated plates and treated with either vehicle (DMSO) or 120 μ M valinomycin for 24 hours. Cells were fixed and stained according to the kit protocol. Treatment with valinomycin results in cell loss, nuclear condensation, increased total nuclear intensity, increased cell permeability, loss of mitochondrial membrane potential and cytochrome c release.

Additional Materials

- Formaldehyde (16%) (Thermo Scientific 16% Formaldehyde, Product No. 28906)
- Collagen I-coated 96-well plates (e.g., BD Biocoat[®] Plates, Product No. 354407)
- Positive control compound such as valinomycin (Sigma Aldrich, Product No. V0627)
- Fetal bovine serum (FBS)

Cell Preparation Information

- This protocol is optimized for HepG2 cells (American Type Culture Collection, Product No. HB-8065). HeLa and A549 cells also have been used successfully in this assay. Using cells other than HepG2 will require protocol optimization.
- For routine culture of cells, use EMEM medium containing the following supplements: 10% fetal bovine serum, 1 mM sodium pyruvate, 1X 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 1:4. Use cells at a passage number \leq 10.
- Harvest cells by trypsinization, dilute into EMEM complete medium and determine cell density. Dilute cells to 7.5×10^4 cells/ml in EMEM complete medium. Add 100 μ l of the cell suspension per well of a 96-well microplate to achieve 7,500 cells/well (recommended plating density is 7,500-15,000 cells/well).
- Incubate cells overnight at 37°C in 5% CO₂ before drug treatment.

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.
Fixation Solution	Add 3 ml of 16% paraformaldehyde solution to 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 44 ml of 1X Wash Buffer. Store this buffer at 4°C for up to 7 days.
Primary Antibody Solution	Add 15 µl of the Cytochrome c Primary Antibody to 6 ml of 1X Blocking Buffer. Prepare solution just before each assay.
Secondary Antibody/Staining Solution	Add 0.6 µl of Hoechst Dye and 12 µl of the DyLight 649 Goat Anti-Mouse to 6 ml of 1X Blocking Buffer. Prepare solution just before each assay.
Live Cell Staining Solution	Add 117 µl of DMSO to the Mitochondrial Membrane Potential Dye to make a 1 mM stock solution. Just before use, add 2.1 µl of Permeability Dye and 21 µl of Mitochondrial Membrane Potential Dye to 6 ml complete medium pre-warmed to 37°C.

B. Procedure

1. Prepare 5X solution of valinomycin (120 µM) and add 25 µl to the cells. Incubate cells at 37°C for 23.5 hours.
2. Add 50 µl of Live Cell Staining Solution to each well.
3. Incubate the cells at 37°C for 30 minutes.
4. Gently aspirate the medium and the staining solution and add 100 µl/well of Fixation Solution and incubate plate for 20 minutes at room temperature.
5. Gently aspirate the Fixation Solution and add 100 µl/well of 1X Wash Buffer.
6. Remove Wash Buffer and add 100 µl/well of 1X Permeabilization Buffer and incubate for 10 minutes at room temperature protected from light.
7. Aspirate Permeabilization Buffer and wash plate twice with 100 µl/well of 1X Wash Buffer.
8. Aspirate Wash Buffer and add 100 µl of 1X Blocking Buffer and incubate for 15 minutes at room temperature.
9. Aspirate Blocking Buffer and add 50 µl/well of Primary Antibody Solution. Incubate for 60 minutes protected from light at room temperature.
10. Aspirate Primary Antibody Solution and wash plate three times with 100 µl/well 1X Wash Buffer.
11. Aspirate Wash Buffer and add 50 µl/well of Secondary Antibody/Staining Solution. Incubate for 60 minutes protected from light at room temperature.
12. Aspirate Secondary Antibody/Staining Solution and wash plate three times with 100 µl/well of 1X Wash Buffer.
13. Add 100 µl/well of 1X Wash Buffer.
14. Seal plate and evaluate on the ArrayScan HCS Reader.
15. Store sealed plates in dark at 4°C. For best results, evaluate plates within 24 hours after assay completion.

Additional Information

A. Dose Response Curves

Changes in nuclear size (in micrometers), total nuclear intensity (percent control), membrane permeability (average nuclear intensity of permeability dye), mitochondrial trans-membrane potential (cytoplasmic intensity of mitochondrial membrane potential dye intensity) and cytochrome c (average intensity of cytoplasmic cytochrome c) were evaluated using the Cellomics Multiparameter Cytotoxicity 3 Kit (Figure 2).

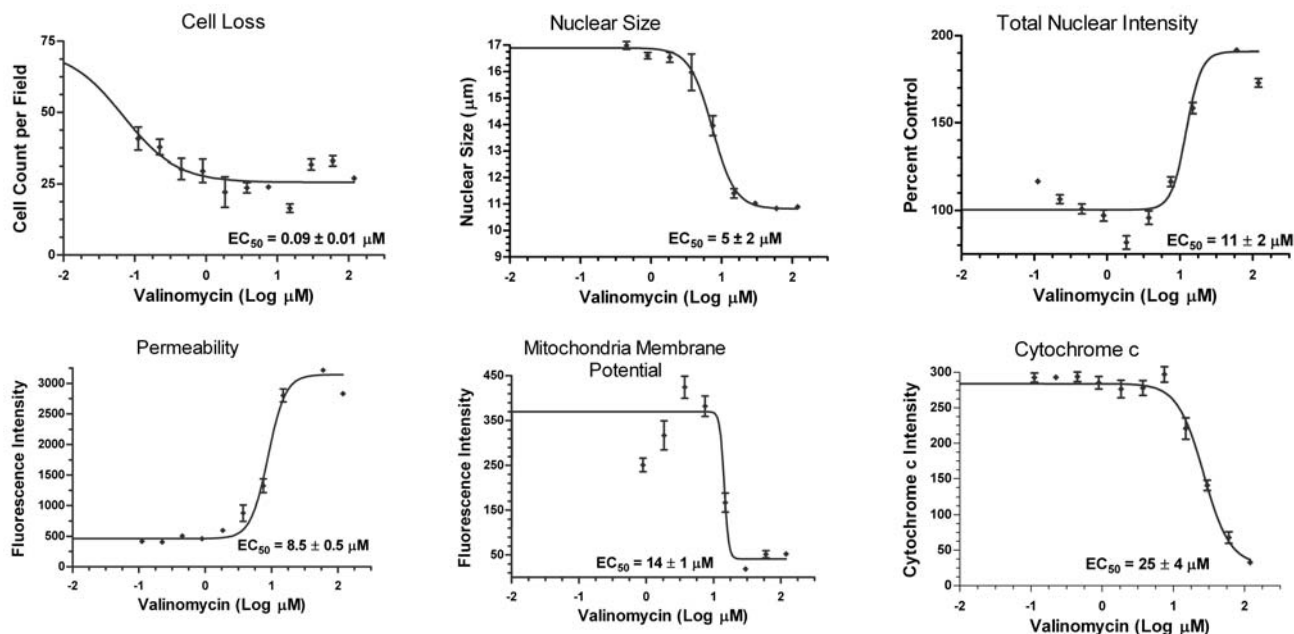


Figure 2. Dose-response curves. HepG2 cells were treated with various doses of valinomycin. Cells were fixed and stained according to the kit protocol.

B. Performance Robustness of the Multiparameter Cytotoxicity 3 Kits

The assay robustness was ascertained by determining the Z' factor for the intensity of the indicated targets in non-treated and valinomycin- (120 μM , 24 hours) treated wells. An effective screening assay produces a positive Z' factor; an excellent screening assay produces a $Z' \geq 0.3$.⁷ The Z' was calculated using three plates of HepG2 cells treated identically. Cell loss and total nuclear intensity were significant using T-test with p-values of 6.2×10^{-29} and 2.6×10^{-38} . The Z' values of other targets are given in the following table:

<u>Target</u>	<u>Z' (mean \pm SD)</u>
Nuclear size	0.50 ± 0.01
Cell permeability	0.60 ± 0.10
Mitochondrial membrane potential	0.80 ± 0.01
Cytochrome c	0.40 ± 0.10

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 649 Conjugates = 646/674 nm

Mitochondrial Membrane Potential Dye = 552/576 nm

Permeability Dye 491/509 nm

Hoechst Dye = 350/461 nm

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 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 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

S50-0022-1 Cell Health Profiling BioApplication

S50-0017-2 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.
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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 or call 800-432-4091 (toll free) or 412-770-2500.

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