Cytochrome C Assay

For High Content Screening

For 5 x 96-well plates

Cat. No. HCS236

FOR RESEARCH USE ONLY
Not for use in diagnostic procedures.

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Introduction

(i) High Content Screening

High Content Screening (HCS) technology offers a major opportunity to improve the drug discovery and development process [1]. HCS enables the evaluation of multiple biochemical and morphological parameters in cellular systems and facilitates characterization of the subcellular distribution of fluorescent signals with labeled reagents. By combining automated imaging of cells with validated detection reagents and powerful image analysis algorithms, scientists can now acquire deeper knowledge of multiple pathways at the single-cell level, usually in a single assay, at an early stage in the development of new drugs [2]. HCS platforms such as the IN Cell Analyzer (GE Healthcare), ArrayScan (ThermoFisher Scientific), or Opera (Perkin Elmer), can be used to deliver detailed profiles of cellular responses [3].

Successful HCS assays rely on high quality reagents [4]. With the commercial availability of thousands of immunoreagents and fluorescent probes, large numbers of fixed-endpoint HCS assays are possible. However, incompatibility of reagents when integrated into a single assay can lead to a significant drop-off in assay performance. Immunoreagents for HCS assays carry special requirements. Strong antigen affinity is required, minimal non-specific binding must be observed, interactions between multiple primary or secondary immunoreagents must be minimized, and the signal to background ratio must be sufficient to ensure an adequate screening window [4]. Additionally, to enable scale up of HCS assays, the sample preparation protocol must be highly reproducible, and the reagents must exhibit minimal assay-to-assay variability.

(ii) Cytochrome C and its significance in cell biology and drug discovery

Cytochrome c is a small heme protein that resides in the mitochondrial intermembrane space [5]. It is primarily known for its function as a key participant in oxidative phosphorylation and ATP synthesis. However, it also plays a key role in regulating apoptosis [5, 6]. When a cell detects an apoptotic stimulus, such as DNA damage or metabolic stress, the intrinsic apoptotic pathway is triggered, and mitochondrial cytochrome c is released into the cytosol, triggering a series of events leading to apoptotic cell death [5]. Cytochrome c is considered a key regulator of apoptosis because once it is released from the mitochondrial intermembrane space, the cell is irreversibly committed to death [7]. Once in the cytosol it binds to Apoptotic protease activating factor-1 (Apaf-1) helping in its oligomerization and the recruitment of procaspase-9 to form a functional apoptosome [8]. Consequently, caspase-9 dissociates from the complex and goes on to activate effector caspases (3, 6, and 7) which cleave specific cellular substrates and collectively orchestrate the execution of apoptosis [6, 7].

Mitochondria play a central role in cancer cell biology because tumor cells rely on glycolysis, but also because evasion of apoptosis is one of the hallmarks of cancer [9]. Given the importance of mitochondrial events during the initial critical stages of the execution of the apoptotic program, targeting mitochondria to induce apoptosis of malignant cells is an important novel therapeutic strategy [7, 10]. In recent years, extensive research has focused on screening for chemical compounds, small molecules and peptides that could target the mitochondria [7]. As a key regulator of apoptosis, cytochrome c release is widely used as a measurement in drug discovery approaches targeting apoptosis.

In the context of anticancer therapy, stimulation of proapoptotic mitochondrial events in tumor cells, and suppression of mitochondrial stages of apoptosis in surrounding normal cells may represent a promising paradigm for new effective therapies [10]. One opportunity is employment of nanoparticles loaded with cell death signals, such as cytochrome c, targeted to cancer cells [10]. Additionally, jasmonates are known to act selectively and directly on cancer cell mitochondria, resulting in membrane depolarization, swelling, and the release of cytochrome c leading to apoptosis of tumor cells [11]. The ultimate goal of these studies is to generate novel mitotoxic agents that can selectively induce apoptosis of cancer cells and reduce the possibility of resistance [7].

The use of microscopy has led to many advances in the field of apoptosis, especially with respect to studying the mitochondrial pathway [6]. The first descriptions of apoptosis described the morphological changes that consistently occur during this form of cell death – namely cell shrinkage, nuclear condensation, blebbing and the formation of apoptotic bodies, each of which are amenable to High Content Screening [6]. Through the use of fluorescent antibody-based detection of cytochrome c, the role and regulation of this important protein in the events that occur during mitochondrial apoptosis may be observed and quantified.

Application

Millipore's HCS236 Cytochrome C Assay provides a complete solution for identifying and quantifying the mitochondrial presence and cytoplasmic release of cytochrome c via cellular imaging. The reagents in the kit have been specifically optimized for HCS applications.

The assay is designed to enable visualization and quantitative detection of cytochrome c, facilitating the identification and characterization of inducers and inhibitors of cytochrome c release. Key applications of this assay include apoptotic pathway analysis, neuroprotection studies, chemotherapeutic efficacy screening and *in vitro* toxicology applications. The nuclear dye (Hoechst 33342) may be used for measurements of cell number, DNA content and nuclear size. Additionally, the assay can be multiplexed with other probes for analysis of proximal cellular events, *e.g.*, for upstream or downstream signaling pathway profiling or for simultaneous screening for cytotoxicity.

The assay is immunofluorescence-based, and utilizes a high quality mouse monoclonal antibody which identifies cytochrome c in human cells. Alternate species cross-reactivity must be confirmed by the end user.

The superior Millipore reagents provided with this kit enable the user to reproducibly generate images with a high signal-to-background ratio, greatly facilitating HCS. In addition, working solutions of the primary and secondary antibodies are stable for at least 24 hours at room temperature (Figure 4), a great benefit for large-scale screening applications. The straightforward sample preparation and processing protocol takes less than 2.5 hrs after fixation. Reagents are provided for 5 x 96-well microplates – *i.e.*, sufficient to perform 480 separate experiments. The kit includes a primary antibody for cytochrome c, a Cy3-conjugated secondary antibody, Hoechst HCS Nuclear Stain, HCS Fixation Solution, HCS Immunofluorescence Buffer, HCS Wash Buffer, and Plate Sealers. Two positive control compounds for cytochrome c release, the antibiotic Staurosporine and the chemotherapeutic agent Paclitaxel, along with DMSO for Compound Serial Dilution and Compound Dilution Buffer, are also included in the kit, sufficient for duplicate 12-point dose response samples per plate (see Assay Instructions). Staurosporine, a broad-spectrum protein kinase inhibitor, is known to induce cytosolic cytochrome c release and apoptosis in a variety of cell types [12]. Paclitaxel, a microtubule stabilization agent, may impact directly on mitochondrial membrane permeability to induce release of cytochrome c [13].

Kit Components

1. Mouse Anti-Cytochrome C HCS Primary Antibody, 100X: - (Part No. CS201731) 1 vial containing 300 µL.
2. HCS Secondary Antibody (donkey anti-mouse IgG, Cy3 conjugate), 200X: - (Part No. CS200437) 1 vial containing 150 µL.
3. Hoechst HCS Nuclear Stain, 200X: - (Part No. CS200438) 1 vial containing 150 µL.
4. HCS Fixation Solution with Phenol Red, 2X: - (Part No. CS200434) 1 bottle containing 100 mL.
5. HCS Immunofluorescence Buffer, 1X: - (Part No. CS200435) 1 bottle containing 1000 mL.
6. HCS Wash Buffer, 1X: - (Part No. 2007643) 1 bottle containing 500 mL.
7. Staurosporine, 2.5 mM in DMSO, 250X: - (Part No. CS201778) 1 vial containing 100 µL.
8. Paclitaxel, 0.25 mM in DMSO, 250X: - (Part No. CS201655) 1 vial containing 100 µL.
9. DMSO for Compound Serial Dilution: - (Part No. CS200441) 1 bottle containing 10 mL.
10. Compound Dilution Buffer: - (Part No. CS200442) 1 bottle containing 25 mL.
11. Plate Sealers: - (Part No. CS200443) 10 each.

Materials Not Supplied

1. Sterile, tissue culture-treated black/clear bottom microplates suitable for High-Content Imaging.
2. Cell-type for assay, *e.g.*, HeLa (human cervical adenocarcinoma, ATCC #CCL-2), A549 (human lung carcinoma, ATCC #CCL-185) or HepG2 (human hepatocellular carcinoma, ATCC #HB-8065).
3. Tissue culture instruments/supplies (including 37ºC incubator, growth media, flasks/plates, detachment buffer, etc.) for cell type of interest.
4. HCS imaging/analysis system, *e.g.*, GE Healthcare IN Cell Analyzer 1000 with Investigator software. System must be equipped with beam-splitters and filters capable of reading emission spectra in the blue and red ranges. Detailed image acquisition and analysis guidelines are provided in Table 2.

Related Products Available from Millipore

19-123MG Staurosporine

Precautions

1. This product contains hazardous materials. Refer to MSDS for further information.

Component	Hazardous Constituent	Warnings (See MSDS)
HCS Fixation Solution	Formaldehyde	Toxic, carcinogen, combustible, readily absorbed through skin
Hoechst HCS Nuclear Stain	Hoechst 33342	Harmful, potential mutagen
Staurosporine	Staurosporine	Toxic, carcinogen
Paclitaxel	Paclitaxel	Harmful, potential mutagen
DMSO	Dimethyl sulfoxide	Combustible, readily absorbed through skin

2. For Research Use Only. Not for use in diagnostic procedures.

Storage

Store kit components under the conditions indicated on the labels. HCS Fixation Solution, HCS Immunofluorescence Buffer, HCS Wash Buffer, DMSO, and Compound Dilution Buffer should be stored at 2-8ºC. Plate Sealers may be stored at room temperature. HCS Primary Antibody, HCS Secondary Antibody, Hoechst HCS Nuclear Stain, Staurosporine and Paclitaxel should be stored at -20ºC, avoiding repeated freeze/thaw cycles. Discard any remaining reagents after 6 months.

(Note: If kit is expected to be used for multiple experiments, rather than a single use, thaw antibodies, nuclear stain and control compound and dispense into appropriately sized aliquots. Store aliquots at -20ºC.)

Assay Instructions

Note: The HCS236 assay protocol has been optimized for HeLa human cervical carcinoma (ATCC #CCL-2), A549 human lung carcinoma (ATCC #CCL-185) and HepG2 human hepatocellular carcinoma (ATCC #HB-8065) cells. However, this kit is suitable for HCS analysis of a variety of human cell types. Alternate species reactivity must be confirmed by the end user.

Cell Preparation:

1. Prior to cell seeding for assay, culture HeLa, A549 or HepG2 cells in growth media until ~70-80% confluent.

2. Detach cells from culture flasks/plates via method appropriate for cell type of interest. If necessary, coat assay plate wells with extracellular matrix protein (*e.g.*, collagen I for HepG2) to enhance cell adhesion. Adjust cell density to 5-7 x 10^4 cells/mL (Hela/A549) or 1-2 x 10^5 cells/mL (HepG2) in growth media. Add 90 µL of this cell suspension to each well (for a 96-well plate, this is approximately equivalent to 15,000-21,000 HeLa or A549 cells/cm^2 of well surface, or 30,000-60,000 HepG2 cells/cm^2). After adding cells to plate, allow plate to sit on a level surface at room temperature for 15-30 min, which allows for even cell distribution. Following this period, incubate cells in growth media (37ºC/5% CO_2) for ~24-48 hours.

3. Cell treatments (control compounds, test compounds, inhibitors, etc.) can be introduced at any point during this culture period, as appropriate for time-course of treatment of interest. Staurosporine and Paclitaxel are provided as known inducers of cytochrome c release and apoptosis. Sufficient reagents are provided for duplicate 12-point dose response curves (including one DMSO-control set within the dose response) for all five 96-well plates. Both compounds are provided at 250X concentration (assuming maximum treatments of 10 µM (staurosporine) and 1 µM (paclitaxel)). Recommended treatment preparation involves half-log (1:√10) serial dilution of the 250X compound in DMSO, followed by dilution in Compound Dilution Buffer to 10X. 10 µL of each treatment may then be added to the 90 µL of culture media already present in each well, for a final 1X concentration (0.4% DMSO). Sample data is provided for 6 (staurosporine) or 24 (paclitaxel) hours of treatment at 37°C prior to fixation.

Cell Fixation and Immunofluorescent Staining:

Note: Staining time is ~2.5 hours post-fixation. Do not allow wells to dry out between staining steps. Aspiration and dispensation of reagents should be conducted at low flow rates to diminish any cell loss due to fluid shear. All recommended 'per well' volumes refer to a single well of a 96-well microplate. All recommended 'per 96-well plate' volumes include 25% excess for liquid handling volume loss. All staining steps are performed at room temperature (RT). All buffers and antibody solutions are stable for at least 24 hours at RT (see Figure 4).

4. At end of culture period, pre-warm HCS Fixation Solution (2X) to room temperature (RT) or 37ºC if desired (12 mL/96-well plate). In a chemical fume hood, add 100 µL/well directly to culture media and allow to fix for 30 min at RT. Remove fixative/toxin-containing media and dispose of in compliance with regulations for hazardous waste (see MSDS). If proceeding immediately to staining, rinse each well twice with 200 µL of HCS Immunofluorescence Buffer. Alternatively, if plates are to be stained at a later time, rinse twice with 200 µL of Wash Buffer, then leave second rinse volume in wells and store plates tightly sealed at 4ºC until staining.

5. If fixed samples have been stored at 4ºC prior to staining, rinse twice with 200 µL HCS Immunofluorescence Buffer before proceeding with staining protocol.

6. Prepare working solution of Mouse Anti-Cytochrome C HCS Primary Antibody (6 mL/96-well plate) as follows: Add 60 µL of thawed Primary Antibody to 5.94 mL of HCS Immunofluorescence Buffer (see Table 1). Mix well. Remove previous Immunofluorescence Buffer rinse. Add 50 µL of Primary Antibody solution to each well and incubate for 1 hour at RT.

7. Remove Primary Antibody solution. Rinse three times with 200 µL HCS Immunofluorescence Buffer.

8. Prepare working solution of Cy3-donkey anti-mouse IgG HCS Secondary Antibody/Hoechst HCS Nuclear Stain (6 mL/96-well plate) as follows: Add 30 µL of thawed Secondary Antibody and 30 µL of thawed Hoechst HCS Nuclear Stain to 5.94 mL of HCS Immunofluorescence Buffer (see Table 1). Mix well, protecting solution from light. Remove previous HCS Immunofluorescence Buffer rinse. Add 50 µL of HCS Secondary Antibody/Hoechst HCS Nuclear Stain solution and incubate for 1 hour at RT, protected from light.

9. Remove HCS Secondary Antibody/Hoechst HCS Nuclear Stain solution. Rinse twice with 200 µL HCS Immunofluorescence Buffer.

10. Remove previous HCS Immunofluorescence Buffer rinse. Rinse twice with 200 µL of HCS Wash Buffer, leaving second rinse volume in wells.

11. Seal plate and image immediately, or store plate at 4ºC protected from light until ready for imaging.

HCS236 Detection Reagent Specifications			
Primary Antibody working solution			
Reagent	**Required dilution of initial reagent**	**Vol. required for 1 well (50 µL)**	**Vol. required for 1 plate (6 mL) (includes ~25% excess)**
Mouse Anti-Cytochrome C HCS Primary Antibody	1:100	0.5 µL	60 µL
HCS Immunofluorescence Buffer	None	49.5 µL	5.94 mL (5940 µL)
Secondary Antibody/Hoechst HCS Nuclear Stain working solution			
Reagent	**Required dilution of initial reagent**	**Vol. required for 1 well (50 µL)**	**Vol. required for 1 plate (6 mL) (includes ~25% excess)**
Cy3-Donkey Anti-Mouse HCS Secondary Antibody	1:200	0.25 µL	30 µL
Hoechst HCS Nuclear Stain	1:200	0.25 µL	30 µL
HCS Immunofluorescence Buffer	None	49.5 µL	5.94 mL (5940 µL)

Table 1. Detection Reagent Specifications – HCS236 Anti-Cytochrome C Assay

Image acquisition and analysis

HCS236 Image Acquisition Guidelines			
Detection Reagent	**Objective Lens**	**Excitation Filter Range [peak/bandwidth (nm)]**	**Emission Filter Range [peak/bandwidth (nm)]**
Hoechst HCS Nuclear Stain	20X	360/40	460/40 (or 535/50 if necessary)
HCS Secondary Antibody, Cy3-donkey anti-mouse IgG	20X	535/50	600/50

HCS236 Image Analysis Guidelines			
Cell Parameter	**Detection**	**Segmentation/ Measurement**	**Rationale**
Cell Number	Hoechst HCS Nuclear Stain	Nuclear region (460 nm emission channel). Count number of nuclei. DNA content (nuclear intensity) or nuclear area analyses are also possible.	Use cell number, nuclear characteristics to determine cell loss, toxicity phenotypes, etc.
Cytochrome C Release	HCS Secondary Antibody, Cy3-conjugated	Nuclear region (600 nm emission channel). Measure Cy3 signal co-localizing with nuclear segmentation. Determine parameters such as average nuclear signal intensity, nuclear:cytoplasmic intensity ratio, etc. Cy3 signal co-localizing with cytoplasmic segmentation may also be analyzed.	Cytochrome c release from the mitochondria results in loss of distinct mitochondrial signal with increased diffuse cytoplasmic and nuclear staining. As “localized” vs. “diffuse” cytoplasmic signal may be difficult to quantify, increases in Cy3 signal visualized within the nuclear-segmented region provide a convenient, sensitive measure of cytochrome c release.

Table 2. Image Acquisition and Analysis Guidelines – HCS236 Anti-Cytochrome C Assay

Sample Results

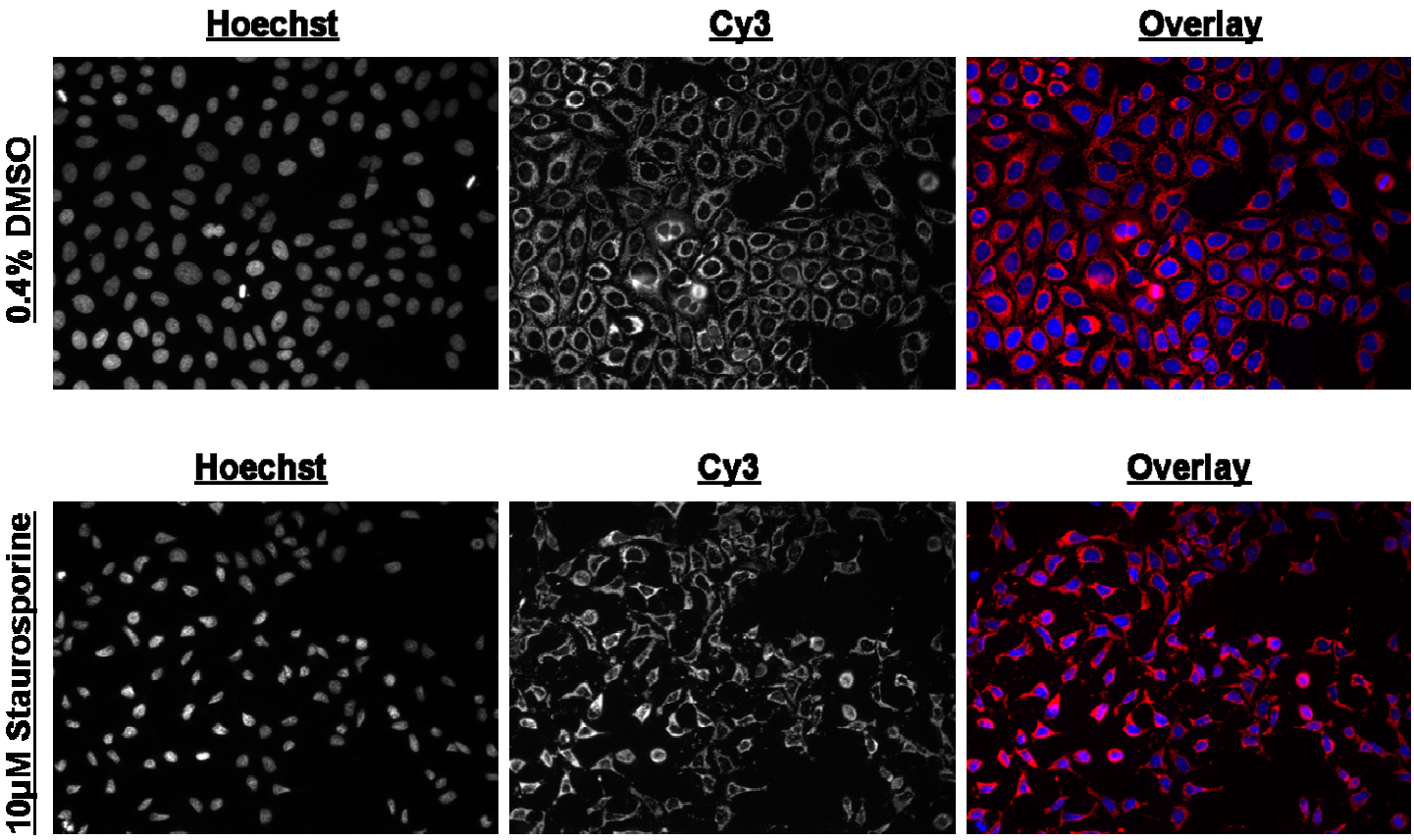

Figure 1. Immunofluorescence of staurosporine-treated HeLa cells.

HeLa cells were plated at 18,000 cells/cm^2 on 96-well plates in growth media and cultured for a total of 48 hours. Cells were treated with 10 µM staurosporine or 0.4% DMSO (negative control) for 6 hours prior to fixation. Cell handling, fixation and immunostaining were performed according to HCS236 assay protocols. Cells were imaged on the GE IN Cell Analyzer 1000 (3.4) at 20X objective magnification. *Left and center panels*: Monochromatic images of Hoechst HCS Nuclear Stain and cytochrome c (Cy3) fluorescence. *Right panel*: Fused images of Hoechst HCS Nuclear Stain (blue) and cytochrome c fluorescence (red).

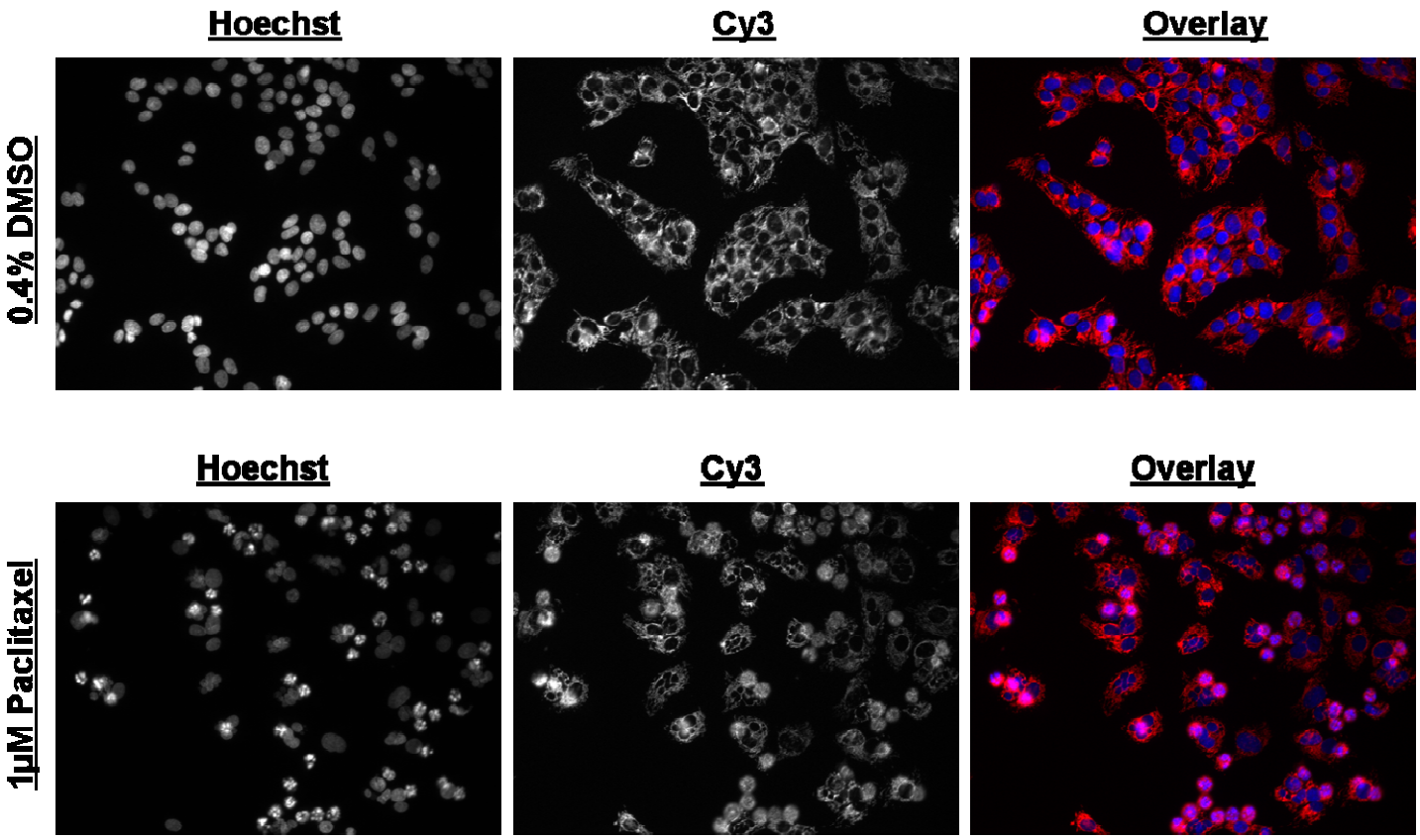

Figure 2. Immunofluorescence of paclitaxel-treated HepG2 cells.

HepG2 cells were plated at 37,000 cells/cm^2 on 96-well plates in growth media and cultured for a total of 48 hours. Cells were treated with 1µM paclitaxel or 0.4% DMSO (negative control) for 24 hours prior to fixation. Cell handling, fixation and immunostaining were performed according to HCS236 assay protocols. Cells were imaged on the GE IN Cell Analyzer 1000 (3.4) at 20X objective magnification. *Left and center panels*: Monochromatic images of Hoechst HCS Nuclear Stain and cytochrome c (Cy3) fluorescence. *Right panel*: Fused images of Hoechst HCS Nuclear Stain (blue) and cytochrome c fluorescence (red).

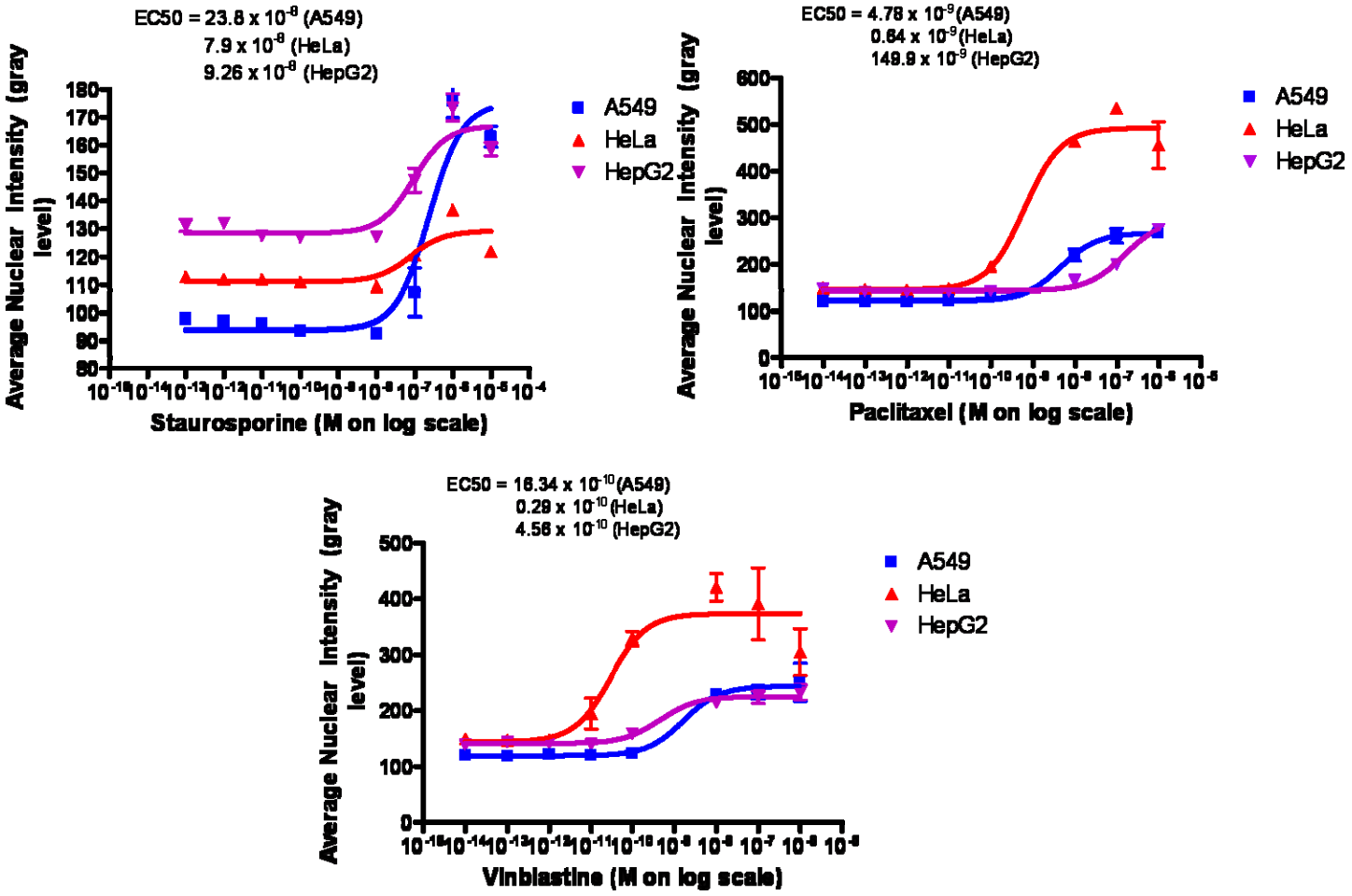

Figure 3. Dose response curves of cytochrome C release in A549, HeLa and HepG2 cells

A549 or HeLa cells were plated at 18,000 cells/cm^2 (37,000 cells/cm^2 for HepG2) on 96-well plates in growth media and cultured for a total of 48 hours. Cells were incubated with serial dilutions of staurosporine (*top left*, 6 hour treatment, max. concentration = 10 µM), paclitaxel (*top right*, 24 hour treatment, max. concentration = 1 µM), or vinblastine (*bottom*, 24 hour treatment, max concentration = 1 µM) prior to fixation. Cell handling, fixation and immunostaining were performed according to HCS236 assay protocols. Cells were imaged on the GE IN Cell Analyzer 1000 (3.4) at 20X magnification (10 fields/well) and analyzed (nuclear/cytoplasmic segmentation) using the GE IN Cell Analyzer 1000 Workstation (3.5) Multi Target Analysis algorithm. Data presented are mean ± SEM, *n* = 3.

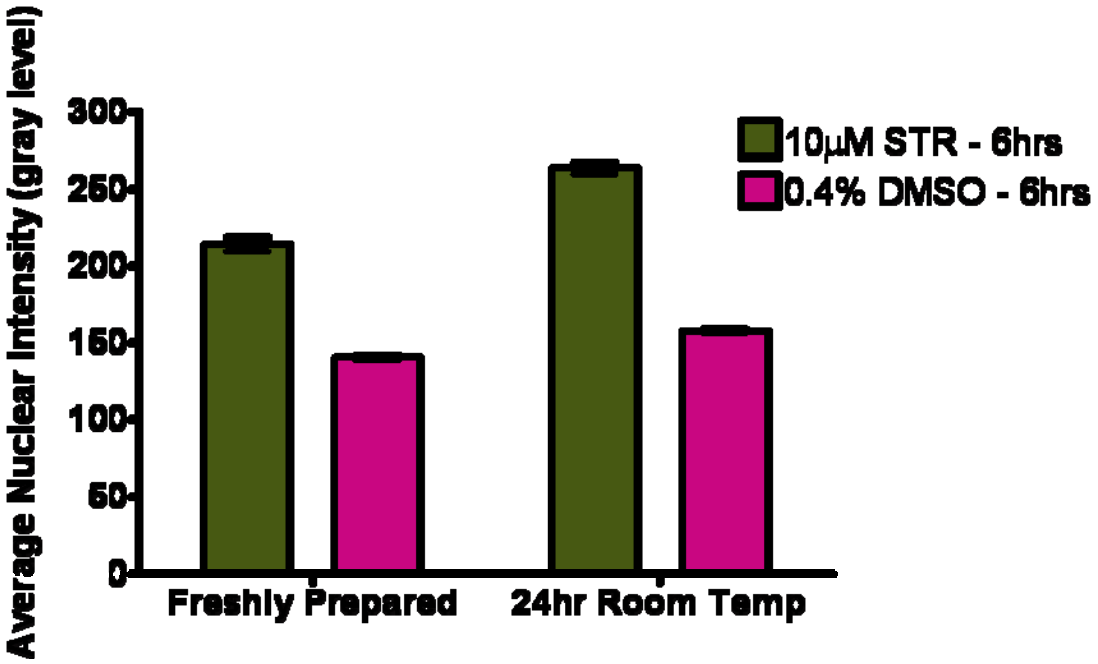

Figure 4. HCS236 Cytochrome C Assay reagent stability.

HeLa cells were plated at 18,000 cells/cm^2 on 96-well plates in growth media and cultured for a total of 48 hours. Cells were treated with 10 µM staurosporine (STR) or 0.4% DMSO (negative control) for 6 hours prior to fixation. Samples were fixed and stained under kit conditions, using either fresh buffers and antibody/Hoechst solutions, or buffers and antibody/Hoechst solutions that had been allowed to sit at room temperature (protected from light) for 24 hours prior to staining. Cells were imaged on the GE IN Cell Analyzer 1000 (3.4) at 20X magnification (10 fields/well) and analyzed (nuclear/cytoplasmic segmentation) using the GE IN Cell Analyzer 1000 Workstation (3.5) Multi Target Analysis algorithm. Average nuclear intensities were measured to observe differences in cytochrome c release between staurosporine and DMSO-treated cells. Data presented are mean ± SEM; *n* = 4. No significant decreases in signal were observed between freshly prepared and 24 hour samples.

Troubleshooting

Problem	Potential Explanations/Solutions
Weak Cy3/Hoechst signal	Improper storage or preparation of Primary/Secondary antibody or Nuclear Stain – retry stain with fresh antibody/dye solution. Inadequate primary/secondary antibody or Nuclear Stain concentrations for cell type – titrate dilutions to optimize signal. Signal may diminish in extremely dense cultures – decrease cell seeding concentration or increase primary/secondary antibody or Hoechst concentration. Optimize exposure times and/or fluorescence filters appropriate to fluorophore.
Excessive background	Improper reagent storage or preparation – retry with fresh reagent (antibodies/dyes and/or buffers). Contaminated buffers/solutions may require 0.2 µm filter sterilization. Samples may have dried during staining – retry stain on fresh samples. Excessive primary or secondary antibody concentrations for cell type – titrate dilutions to optimize signal. Check for autofluorescence of microplate.
Excessive Cy3/ Hoechst signal	Improper preparation of antibody/dye – retry stain with fresh antibody/dye solutions. Inappropriate antibody/dye concentrations for cell type – titrate dilutions to optimize signal. Optimize exposure times and/or fluorescence filters appropriate to fluorophore.
Cell loss	Optimize liquid aspiration/dispensation rate to reduce shear. Consider protein-coating to improve cell adhesion to microplate. Optimize cell seeding concentrations for better cell adhesion. Cell loss due to toxic treatments may hinder statistically relevant analysis; alter toxin dosages/treatment times to reduce cell loss levels.
Poor nuclear/cytoplasmic segmentation during analysis	Effective segmentation parameters can be HCS system/software-dependent. Consider decreasing cell seeding concentrations for difficulty in analysis of dense cultures (separation of multiple nuclei).

Problem	Potential Explanations/Solutions
No dose response observed/partial response curve	Efficacy of control compounds may vary with cell type, cell species, or quality of reagent storage. Use fresh compound, choose alternate maximum/minimum treatment concentrations, or select more appropriate control compounds for cell type of interest. Perform time-course experiments to determine kinetics of compound effects for cell type of interest. Shorter/longer treatment durations may be required.

References

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