Hepatotoxicity Assay, Human HepG2 Cells

Catalog No. HCS100

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Not for use in diagnostic procedures

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Introduction

(i) High Content Screening, Cellular Systems Biology and Drug Discovery

The current drug development process is slow, and results in a fail rate exceeding 90%. The development of High Content Screening (HCS) technology represented a major step towards improving the drug discovery and development process [1]. High Content Screening enables the evaluation of several biochemical and morphological parameters in cellular systems. By combining the automated imaging of cells in microtiter plates with high-quality detection reagents and powerful image analysis algorithms, scientists can acquire deeper knowledge of several morphological or biochemical pathways at the single-cell level at an early stage in the development of new drugs. HCS platforms such as the IN Cell Analyzer (GE Healthcare), ArrayScan (ThermoFisher Scientific), or Opera (Perkin Elmer), when coupled with panels of reagents and informatics tools, can be used to deliver detailed profiles of cellular systemic responses, rather than merely observing a subset of cellular events [2].

With the commercial availability of thousands of immunoreagents and fluorescent probes, large numbers of multiplexed fixed-endpoint HCS assays are possible. However, incompatibility of reagents when integrated into a single multiplexed assay can lead to a significant drop-off in assay performance. Furthermore, multiplexed assays involving the use of a panel of immunoreagents have an inherent complexity that demands strict attention to detail to achieve a validated assay for HCS [3]. Immunoreagents for multiplexed 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. Additionally, to enable scale up of validated multiplexed HCS assays, the sample preparation protocol must be highly reproducible and compatible with automation.

(ii) Hepatotoxicity Profiling using High Content Screening Platforms

Drug-induced hepatotoxicity is a major contributor to high attrition rates during preclinical and clinical drug development, and has even resulted in the withdrawal of approved drugs from the market [4, 5]. Detection of human hepatotoxicity using traditional *in vitro* studies has been unreliable, due to poor assay and/or reagent specificity, insufficient numbers of endpoints, and an inability to detect early stages of hepatotoxicity [4]. Consequently, there is an acute need for improved *in vitro* hepatotoxicity assays.

In the drug discovery process, the use of HCS platforms for measurement of the effects of potential lead compounds on multiple cellular pathways has become a valuable tool, especially when applied early on in the discovery process [6]. Notably, HCS has been demonstrated to be an effective tool for determination of drug-induced human hepatotoxicity [4] using the human hepatocellular carcinoma cell line HepG2, a widely used cellular model for *in vitro* cytotoxicity studies [7, 8]. Recent data indicate that multiparametric High Content Screening for hepatotoxicity using human HepG2 cells is expected to be a more reliable indicator of human hepatotoxicity than animal models [4].

Accordingly, Millipore has developed a Hepatotoxicity Assay for human HepG2 cells, a multiplexed HCS kit comprised of high-quality, validated, automation-compatible detection reagents and validated protocols for profiling multiple human hepatotoxicity endpoints. This assay provides a means to screen compounds for a broad range of potentially toxic effects early in the drug discovery process, and provide better information to drive drug development.

Application

Millipore's HCS100 Hepatotoxicity Assay for human HepG2 cells is a kit for multiparametric analysis of drug-induced human hepatotoxicity. Parameters were selected to monitor the effects of test compounds on many cellular systems responses known to be correlated with toxic challenge. Eleven cellular responses may be analyzed using this assay kit.

1. Cell Loss.
2. Cell Cycle Arrest.
3. DNA Degradation/Apoptosis.
4. Nuclear Size.
5. Oxidative Stress.
6. Stress Pathway Activation.
7. DNA Damage Response.
8. Mitochondrial Membrane Potential.
9. Mitochondrial Mass.
10. Mitotic Arrest.
11. Cytoskeletal Integrity.

The assay has been designed to allow detection of all 11 parameters at three separate time points (*e.g.*, acute – 1 hr, early – 24 hr, chronic – 72 hr). Reagents and materials supplied in the HCS100 Hepatotoxicity Kit are sufficient to perform ten-point dose response curves in duplicate for four control toxins (provided) and sixteen unknown compounds at each time point. The kit also includes buffers for Compound Dilution, Fixation, Cell Permeabilization, Antibody Dilution and Washing, as well as disposable syringes and needles for HepG2 cell handling and plate sealers. All materials and protocols provided have been validated for multiplexed analysis on both the GE IN Cell Analyzer 1000 and the ThermoFisher ArrayScan.

The assay has been designed to be performed in 6 tissue-culture treated 384-well microplates, with two plates required for each timepoint. See Table 1 below for multiplexed plate layout. Note: Plates with different well numbers (*e.g.,* 96 or 1536 wells) may be used by making appropriate volume adjustments.

Table 1. Assay profiles by plate, showing labels and features.

PLATE # 1		
Cell Parameter	**Measurement**	**Detection Reagents**
1. Cell Loss	Cell number	Hoechst HCS Nuclear Stain
2. Cell Cycle Arrest	DNA content	Hoechst HCS Nuclear Stain
3. DNA Degradation/ Apoptosis	DNA content/ morphology	Hoechst HCS Nuclear Stain
4. Nuclear Size	Nuclear area	Hoechst HCS Nuclear Stain
5. Oxidative Stress	Phosphorylation	Antibodies A & B. *Detection of Phospho-Histone H2A.X*$^{(Ser139)}$
6. Stress Pathway Activation	Phosphorylation	Antibodies C & D. *Detection of Phospho-c-jun*$^{(Ser73)}$
7. DNA Damage Response	Target activation	Antibodies E & F. *Detection of p53*
PLATE #2		
Cell Parameter	**Measurement**	**Detection Reagents**
Cell Loss	Cell number	Hoechst HCS Nuclear Stain
Cell Cycle Arrest	DNA content	Hoechst HCS Nuclear Stain
DNA Degradation/ Apoptosis	DNA content/ morphology	Hoechst HCS Nuclear Stain
Nuclear Size	Nuclear area	Hoechst HCS Nuclear Stain
8. Mitochondrial Membrane Potential	Mitochondrial membrane potential	MitoDye
9. Mitochondrial Mass	Mitochondrial mass	MitoDye
10. Mitotic Arrest	Phosphorylation	Antibodies G & H. *Detection of Phospho-Histone H3*$^{(Ser10)}$
11. Cytoskeletal Integrity	Microtubule stability	Antibodies I & J. *Detection of α-tubulin*

Kit Components

1. Antibody A, 100x. Anti-phospho-histone H2A.X: (Part No. 2007523) 1 vial containing 200 µL.
2. Antibody B, 200x. Anti-Antibody A, FITC conjugate: (Part No. 2007528) 1 vial containing 100 µL.
3. Antibody C, 100x. Anti-phospho-c-jun: (Part No. 2007524) 1 vial containing 200 µL.
4. Antibody D, 200x. Anti-Antibody C, Cy3 conjugate: (Part No. 2007530) 1 vial containing 100 µL.
5. Antibody E, 100x. Anti-p53: (Part No. 2007525) 1 vial containing 200 µL.
6. Antibody F, 200x. Anti-Antibody E, Cy5 conjugate: (Part No. 2007531) 1 vial containing 100 µL.
7. Antibody G, 100x. Anti-phospho-histone H3: (Part No. 2007526) 1 vial containing 200 µL.
8. Antibody H, 200x. Anti-Antibody G, FITC conjugate: (Part No. 2007532) 1 vial containing 100 µL.
9. Antibody I, 100x. Anti-α-tubulin: (Part No. 2007527) 1 vial containing 200 µL.
10. Antibody J, 200x. Anti-Antibody I, Cy5 conjugate: (Part No. 2007533) 1 vial containing 100 µL.
11. MitoDye solution, 10,000x: (Part No. 2007516) 1 vial containing 25 µL
12. Hoechst HCS Nuclear Stain, 200x: (Part No. 2007515) 1 vial containing 200 µL.
13. Compound Dilution Buffer: (Part No. 2007512) 1 bottle containing 500 mL.
14. HCS Fixation Solution with Phenol Red, 2x: (Part No. 2007513) 1 bottle containing 200 mL.
15. Cell Permeabilization Buffer: (Part No. 2007514) 1 bottle containing 100 mL.
16. Antibody Dilution Buffer: (Part No. 2007511) 1 bottle containing 100 mL.
17. Wash Buffer: (Part No. 2007534) 2 bottles each containing 1000 mL.
18. Anisomycin, 2.5 mM in DMSO, 250x: (Part No. 2007477) 1 vial containing 100 µL.
19. Camptothecin, 2.5 mM in DMSO, 250x: (Part No. CS201666) 1 vial containing 100 µL.
20. CCCP, 25 mM in DMSO, 250x: (Part No. 2007479) 1 vial containing 100 µL.
21. Paclitaxel, 0.25 mM in DMSO, 250x: (Part No. CS201655) 1 vial containing 100 µL.
22. 20 gauge needle with syringe, 5cc capacity: (Part No. 2007517) 6 each.
23. Plate Sealers: (Part No. CS200443) 10 each.

Materials Not Supplied

1. HepG2 cells. Available from ATCC, catalog number HB-8065.
2. Tissue culture equipment/supplies for culturing HepG2 cells (*e.g.*, 37ºC incubator, cell culture hood, growth media, Trypsin-EDTA solution, tissue culture flasks/plates, centrifuge tubes, pipettes, etc).
3. 384-well microplates suitable for High Content Imaging of HepG2 cells.
4. Dimethyl sulfoxide (DMSO). HPLC grade is recommended.
5. High Content Imaging hardware and software, *e.g.*, GE Healthcare IN Cell Analyzer 1000 with Investigator software. Imaging system must be equipped with beam-splitters and filters capable of reading emission spectra in the blue, green, red and far red ranges. See Table 2 for example filter ranges.

Table 2. Recommended filter ranges for HCS100 imaging

Fluorescent Reagent	Excitation Filter Range [peak/bandwidth (nm)]	Emission Filter Range [peak/bandwidth (nm)]
Hoechst HCS Nuclear Stain	360/40	460/40 (or 535/50 if necessary)
FITC	480/40	535/50
Cy3/MitoDye	535/50	600/50
Cy5	620/60	700/75

Storage

Store kit components under the conditions indicated on the labels. Compound Dilution Buffer, HCS Fixation Solution, Cell Permeabilization Buffer, Antibody Dilution Buffer and Wash Buffer should be stored at 2-8ºC. 20G needle/syringes and plate sealers may be stored at room temperature. All other components should be stored at -20ºC. Compound Dilution Buffer and the four Control Toxins should be handled under aseptic conditions for best results. Discard any remaining reagents after 6 months.

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
Anisomycin in DMSO	Anisomycin	Toxic
Camptothecin in DMSO	Camptothecin	Toxic
CCCP in DMSO	CCCP	Harmful
Paclitaxel in DMSO	Paclitaxel	Harmful, potential mutagen
DMSO	Dimethyl sulfoxide	Combustible, readily absorbed through skin

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

Assay Instructions

Assay Summary

a. Plate HepG2 cells on 384-well microplates and incubate for 18-24 hr.

b. Treat cells with positive control toxins (provided) and test compounds.

i. Camptothecin: 10-point dose response, Maximum final conc. = 10 µM

ii. Anisomycin: 10-point dose response, Maximum final conc. = 10 µM

iii. CCCP: 10-point dose response, Maximum final conc. = 100 µM

iv. Paclitaxel: 10-point dose response, Maximum final conc. = 1 µM

c. Label cells with physiological indicator dyes (Hoechst HCS Nuclear Stain and MitoDye), primary antibodies and corresponding secondary antibodies.

d. Perform imaging and analysis.

1. Assay and Reagent Specifications

1.1 Table 1 contains the labels and features of Multiplex Plates 1 and 2.

1.2 Table 2 contains recommended filter ranges

1.3 Table 3 contains MitoDye reagent specifications (for Multiplex Plate 2).

1.4 Table 4 contains antibody and fluorescent indicators of cell physiology reagent specifications for Multiplex Plate 1.

1.5 Table 5 contains antibody and fluorescent indicators of cell physiology reagent specifications for Multiplex Plate 2.

1.6 Table 6 contains control toxin (anisomycin, camptothecin, CCCP, paclitaxel) specifications.

1.7 Table 7 contains image acquisition setup recommendations

1.8 Table 8 contains recommended image analysis measurements

1.9 Table 9 contains representative AC_{50} values of selected cell features

2. Assay Protocols

2.1 Recommended HepG2 cell handling and plating procedure

2.1.1 Obtain HepG2 cells from ATCC (cat no. HB-8065).

2.1.2 Cells should be maintained in HepG2 growth medium according to ATCC instructions – Eagle's Minimum Essential Medium (MEM) with 2 mM L-glutamine and Earle's BSS adjusted to contain 1.5 g/L sodium bicarbonate, 0.1 mM non-essential amino acids, and 1.0 mM sodium pyruvate, supplemented with 10% fetal bovine serum. Penicillin-streptomycin may also be added to culture media.

2.1.3 Maintain cells in T-150, vented, uncoated TC flasks using 20 ml culture medium. Culture in a 37℃ incubator with 5% CO_2.

2.1.4 Make cell passages at 1:4 or 1:5 (approximately 4 x 10^6 cells) every 3-4 days when cells are ~70% confluent.

2.1.4.1 Aspirate medium from a T-150 flask of HepG2 cells.

2.1.4.2 Wash monolayer 2 times with 4 mL prewarmed 0.25% trypsin with EDTA.

2.1.4.3 Add 2 mL trypsin.

2.1.4.4 Trypsinize for 10 min at 37ºC until the monolayer lifts. Add 8mL growth medium to flask to deactivate trypsin.

2.1.4.5 Pipet up and down several times to reduce clumps.

2.1.4.6 Transfer cells to a 15 mL centrifuge tube.

2.1.4.7 Carefully triturate using a 20G needle with 5 ml syringe (provided) three times until clumps of cells are reduced.

2.1.4.8 Count and dilute cells as required.

2.1.5 OPTIONAL STEP - synchronization of HepG2 cells

2.1.5.1 The day prior to plating cells into microplates, passage HepG2 cells (~70% confluent) by trypsinization, including trituration using 20G needle with 5 ml syringe (provided), and replate into the same flask from which they were removed. This optional step is designed to ensure that each assay is performed using HepG2 cells in a similar phase of growth.

2.1.6 Cell plating

2.1.6.1 For this assay, Millipore recommends using collagen I-coated thin bottom 384-well microplates that are compatible with HCS readers.

2.1.6.2 Cells are passaged by trypsinization, including trituration, and viable cells counted.

2.1.6.3 Prepare 20 mL of cell suspension per plate at concentrations of 1.0, 2.0 and 3.5 x 10^6 cells/20 mL (5 X 10^4, 1 X 10^5 , 1.75 X 10^5 cells/ml). 40 µL of cell suspension is plated into each well to yield the following cell densities for each time point:

- 1 hr treatment: 7000 cells per well.
- 24 hr treatment: 4000 cells per well.
- 72 hr treatment: 2000 cells per well.

2.1.6.4. After each plate is filled, place onto a stable benchtop to settle for 30 min. This ensures even cellular distribution over the surface of each well. Begin timing after last plate in batch is placed on the benchtop.

2.1.6.5 After 30 min settling at room temperature, place the plates into a 37ºC/5% CO_2 incubator.

2.1.6.6 Allow cells to attach and spread for 18-24 hr before compound addition.

2.2 Compound preparation and treatment of cells

2.2.1 4 positive controls (See Table 6 for further details) are provided in a 250x formulation in DMSO at the following concentrations:

- Anisomycin: 2.5 mM (Maximum final conc. = 10 µM)
- Camptothecin: 2.5 mM (Maximum final conc. = 10 µM)
- CCCP: 25 mM (Maximum final conc. = 100 µM)
- Paclitaxel: 0.25 mM (Maximum final conc. = 1 µM)

2.2.2 Test/unknown compounds should be prepared in DMSO at concentrations up to 25 mM and stored at -20 C.

2.2.3 A 10-point half-log dilution set is made for each compound by diluting slightly more than 3-fold (square root of 10) on each step. This simplifies display of resulting data on a log scale. To promote solubility, it is recommended to perform an initial 10-point serial dilution set of 250x compounds in DMSO before diluting each to 5x in Compound Dilution Buffer (provided). For example:

- Anisomycin: 10 µM maximum final concentration. For each 3 plate set, start with 50 µL of 250x stock (2.5mM). Serially dilute by sequentially adding 16 µL of compound to 34 µL of DMSO to make each subsequent dilution. Once the 10-point serial dilution of 250x compound in DMSO is completed, dilute to make 200µL per concentration of 5x compound (50 µM maximum) in Compound Dilution Buffer (4 µL of 250x into 196 µL Buffer).
- Camptothecin: Prepare as above. 10 µM maximum final concentration. For each 3 plate set, prepare 200 µL of a 5x solution per concentration (50 µM maximum) in Compound Dilution Buffer.
- CCCP: Prepare as above. 100 µM maximum final concentration. For each 3 plate set, prepare 200 µL of a 5x solution per concentration (500 µM maximum) in Compound Dilution Buffer.
- Paclitaxel: Prepare as above. 1 µM maximum final concentration. For each 3 plate set, prepare 200 µL of a 5x solution per concentration (5 µM maximum) in Compound Dilution Buffer.

- DMSO Controls: Prepare 5 mL of 5x (*i.e.* 2%) solution for each 6 plate set (add 100 µL of DMSO to 4.9 mL of Compound Dilution Buffer).

2.2.4 Compound additions are made by transferring 10 µL of 5x compound stocks to each well, giving 50 µL total well volume and concentrations of 1x.

2.2.4.1 According to this scheme, DMSO is used at a maximal final concentration of 0.4% in each well after compound addition (50 µL total volume).

2.2.4 2 After each plate is filled, return plates to 37ºC/5% CO_2 incubator. Begin timing of incubation after last plate in batch is placed in incubator.

2.3. Labeling of Multiplex Plate 2 with MitoDye - performed just prior to fixation

2.3.1 Prepare MitoDye working solution (2x). This is done by diluting the 10,000x MitoDye stock solution (provided) 1:5,000 in warmed HepG2 growth medium for a final 2x concentration, *e.g.*, 5 µL of 10,000x MitoDye stock solution diluted in 25 mL of HepG2 growth medium gives 25 mL of MitoDye working solution. See Table 3 below. Approximately 25 mL of MitoDye working solution is needed per plate, although this may vary according to each specific user's requirements.

2.3.2 Gently add 50 µL of this 2x MitoDye working solution to each well. (Note: do not remove the 50 µL of growth/compound medium from well).

2.3.3 Incubate the plate for 15 min at 37ºC in 5% CO_2 incubator.

2.3.4 Remove fluid from the plate.

2.3.5 Gently add 50 µL of HepG2 growth medium to each well.

2.3.6 Proceed with cell fixation protocol.

Table 3. Preparation of MitoDye working solution for Multiplex Plate 2.

PREPARATION OF WORKING MITODYE SOLUTION FOR MULTIPLEX PLATE 2.				
Cell Parameter	**Reagent**	**Required dilution of initial reagent**	**Vol. required for 50 µL (1 well) MitoDye working solution**	**Vol. required for 25 mL (1 plate) MitoDye working solution**
8-9. Mitochondrial Membrane Potential/ Mitochondrial Mass	MitoDye, 10,000x	1:5,000	0.01 µL	5 µL
	Warmed HepG2 growth medium	None	49.99 µL	25 mL (24,995 µL)

2.4 Cell fixation

2.4.1 HCS Fixation Solution (2x) should be added to wells containing 50 µL of HepG2 growth medium, *i.e.*, do not remove growth medium prior to fixation.

2.4.2 In a chemical fume hood, gently add 50 µL HCS Fixation Solution (2x) to each well.

2.4.3 Incubate plates in fume hood for 30 minutes at room temperature. Protect from light (in order to maintain MitoDye fluorescence).

2.4.4 Wash cells with 100 µL/well Wash Buffer and immediately remove.

2.4.5 Proceed with cell permeabilization and labeling protocol. Alternatively, if plates are to be stored before labeling, re-fill wells with 100 µL/well Wash Buffer, seal tightly with Plate Sealers and store at 4℃ protected from light . Wells containing MitoDye should ideally be imaged within 48 hr of dye loading.

2.5. Cell permeabilization and labeling protocol

2.5.1 Prepare Multiplex Plate 1 and Plate 2 Primary Reagents Cocktail as indicated in Table 4. Approximately 5 mL per plate are needed, although this may vary according to each specific user's requirements.

2.5.2 Permeabilize cells by adding 16 µL/well Cell Permeabilization Buffer. Incubate for 5 minutes at room temperature, protected from light. Do not remove.

2.5.3 Wash cells with 100 µL/well Wash Buffer and immediately remove.

2.5.4 Add 10 µL/well of Multiplex Plate 1 Primary Reagents Cocktail to each well in Plate 1 and incubate for 1 hour at room temperature, protected from light.

2.5.5 Add 10 µL/well of Multiplex Plate 2 Primary Reagents Cocktail to each well in Plate 2 and incubate for 1 hour at room temperature, protected from light.

2.5.6 Prepare Multiplex Plate 1 and Plate 2 Secondary Reagents Cocktail as indicated in Table 5. Approximately 5 mL per plate are needed, although this may vary according to each specific user's requirements.

2.5.7 Wash wells with 100 µL/well Wash Buffer and immediately remove.

2.5.8 Add 10 µL/well of Multiplex Plate 1 Secondary Reagents Cocktail to each well in Plate 1 and incubate for 1 hour at room temperature, protected from light.

2.5.9 Add 10 µL/well of Multiplex Plate 2 Secondary Reagents Cocktail to each well in Plate 2 and incubate for 1 hour at room temperature, protected from light.

2.5.10 Wash cells 2x with 100 µL/well Wash Buffer, leaving the second wash in the wells.

2.5.11 Seal plates for imaging and analysis, using Plate Sealers provided. Plates can be stored, protected from light, at 4ºC for up to 48 hours before imaging.

Table 4. Detection reagent requirements for Multiplex Plate 1

MULTIPLEX PLATE 1 – PRIMARY REAGENTS COCKTAIL				
Cell Parameter	**Reagent**	**Required dilution of initial reagent**	**Volume for 10 µL (1 well) reagent cocktail**	**Volume for 5 mL (1 plate) reagent cocktail**
5. Oxidative Stress	Antibody A. Anti-Phospho-Histone H2A.X	1:100	0.1 µL	50 µL
6. Stress Pathway Activation	Antibody C. Anti-Phospho-c-jun	1:100	0.1 µL	50 µL
7. DNA Damage Response	Antibody E. Anti-p53	1:100	0.1 µL	50 µL
	Antibody Dilution Buffer	None	9.7 µL	4.85 mL (4850 µL)
MULTIPLEX PLATE 1 – SECONDARY REAGENTS COCKTAIL				
Cell Parameter	**Reagent**	**Required dilution of initial reagent**	**Volume for 10 µL (1 well) reagent cocktail**	**Volume for 5 mL (1 plate) reagent cocktail**
1-4. Nucleus	Nuclear Stain	1:200	0.05 µL	25 µL
5.Oxidative Stress	Antibody B. FITC-anti-Antibody A	1:200	0.05 µL	25 µL
6. Stress Pathway Activation	Antibody D. Cy3-anti-Antibody C	1:200	0.05 µL	25 µL
7. DNA Damage Response	Antibody F. Cy5-anti-Antibody E	1:200	0.05 µL	25 µL
	Antibody Dilution Buffer	None	9.8 µL	4.9mL (4900 µL)

Table 5. Detection reagent requirements for Multiplex Plate 2

MULTIPLEX PLATE 2 – PRIMARY REAGENTS COCKTAIL				
Cell Parameter	**Reagent**	**Required dilution of initial reagent**	**Volume for 10 µL (1 well) reagent cocktail**	**Volume for 5 mL (1 plate) reagent cocktail**
10. Mitotic Arrest	Antibody G. Anti-Phospho-Histone H3	1:100	0.1 µL	50 µL
11. Cytoskeletal Integrity	Antibody I. Anti-α-tubulin	1:100	0.1 µL	50 µL
	Antibody Dilution Buffer	None	9.8 µL	4.9 mL (4900 µL)
MULTIPLEX PLATE 2 – SECONDARY REAGENTS COCKTAIL				
Cell Parameter	**Reagent**	**Required dilution of initial reagent**	**Volume for 10 µL (1 well) reagent cocktail**	**Volume for 5 mL (1 plate) reagent cocktail**
Nucleus	Nuclear Stain	1:200	0.05 µL	25 µL
10. Mitotic Arrest	Antibody H. FITC-anti-Antibody G	1:200	0.05 µL	25 µL
11. Cytoskeletal Integrity	Antibody J. Cy5-anti-Antibody I	1:200	0.05 µL	25 µL
	Antibody Dilution Buffer	None	9.85 µL	4.925 mL (4925 µL)

Table 6. Positive control toxins

Cell Parameter	Positive Control	Concentration Supplied	Maximum Final Concentration
1. Cell Loss	Paclitaxel	0.25 mM	1.0 µM
2. Cell Cycle Arrest	Camptothecin	2.5 mM	10 µM
3. DNA Degradation/ Apoptosis	Paclitaxel	0.25 mM	1 µM
4. Nuclear Size	Camptothecin	2.5 mM	10 µM
5. Oxidative Stress	Anisomycin	2.5 mM	10 µM
6. Stress Pathway Activation	Anisomycin	2.5 mM	10 µM
7. DNA Damage Response	Camptothecin	2.5 mM	10 µM
8. Mitochondrial Membrane Potential	CCCP	25 mM	100 µM
9. Mitochondrial Mass	CCCP	25 mM	100 µM
10. Mitotic Arrest	Paclitaxel	0.25 mM	1 µM
11. Cytoskeletal Integrity	Paclitaxel	0.25 mM	1 µM

Suggested example of layouts for Multiplex Plates 1 and 2. Plate layouts may vary according to each specific user's requirements.

1. Each plate to contain 24 DMSO control wells distributed in the corners.
2. Each plate to contain 2 duplicate positive control toxin 10-point responses (minimum concentration in row D, maximum in row M)
3. Each plate to contain 16 duplicate test toxin 10-point responses (minimum concentrations in columns 3 and 12)
4. Suggested plate layout scheme is shown in Figure 1 below.

Figure 1. Suggested layouts for Multiplex Plates 1 and 2.

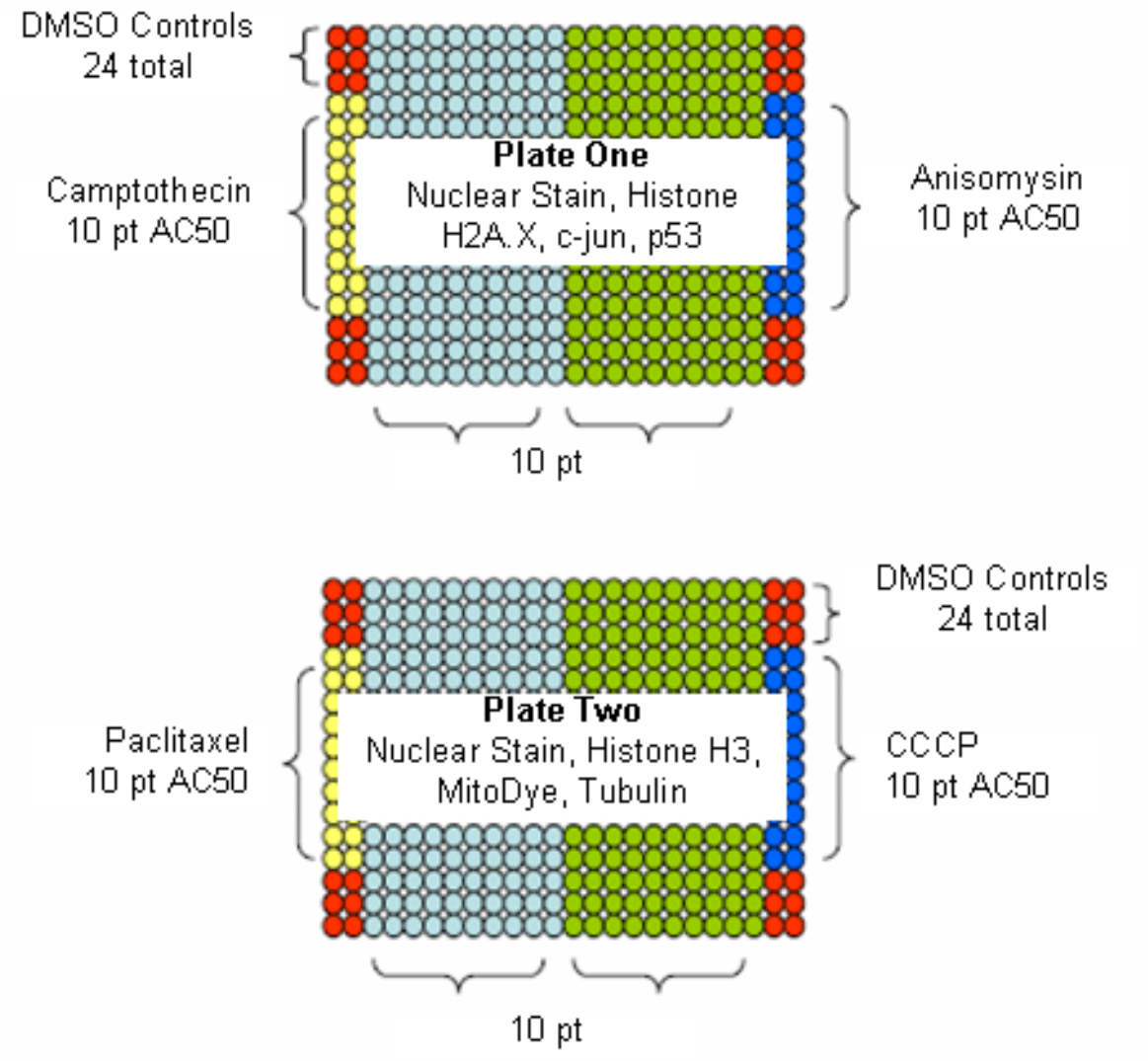


Image Acquisition

A recommended image acquisition setup is given below. Image collection is suggested using a 10X objective lens, collecting at least 1500 cells per well for parameter analysis (approximately 6-12 fields per well at 10X).

Table 7. Recommended image acquisition setup

Cell Parameter	**Excitation /Emission Wavelengths (nm)**
1. Cell Loss (Hoechst HCS Nuclear Stain)	355/465
2. Cell Cycle Arrest (Hoechst HCS Nuclear Stain)	355/465
3. DNA Degradation/Apoptosis (Hoechst HCS Nuclear Stain)	355/465
4. Nuclear Size (Hoechst HCS Nuclear Stain)	355/465
5. Oxidative Stress (FITC-Phospho-Histone H2A.X)	492/520
6. Stress Pathway Activation (Cy3-Phospho-c-jun)	550/570
7. DNA Damage Response (Cy5-p53)	650/680
8. Mitochondrial Membrane Potential (MitoDye)	579/599
9. Mitochondrial Mass (MitoDye)	579/599
10. Mitotic Arrest (FITC-Phospho-Histone H3)	492/520
11. Cytoskeletal Integrity (Cy5-α-Tubulin)	650/680

Image Analysis

Table 8. Recommended image analysis measurements

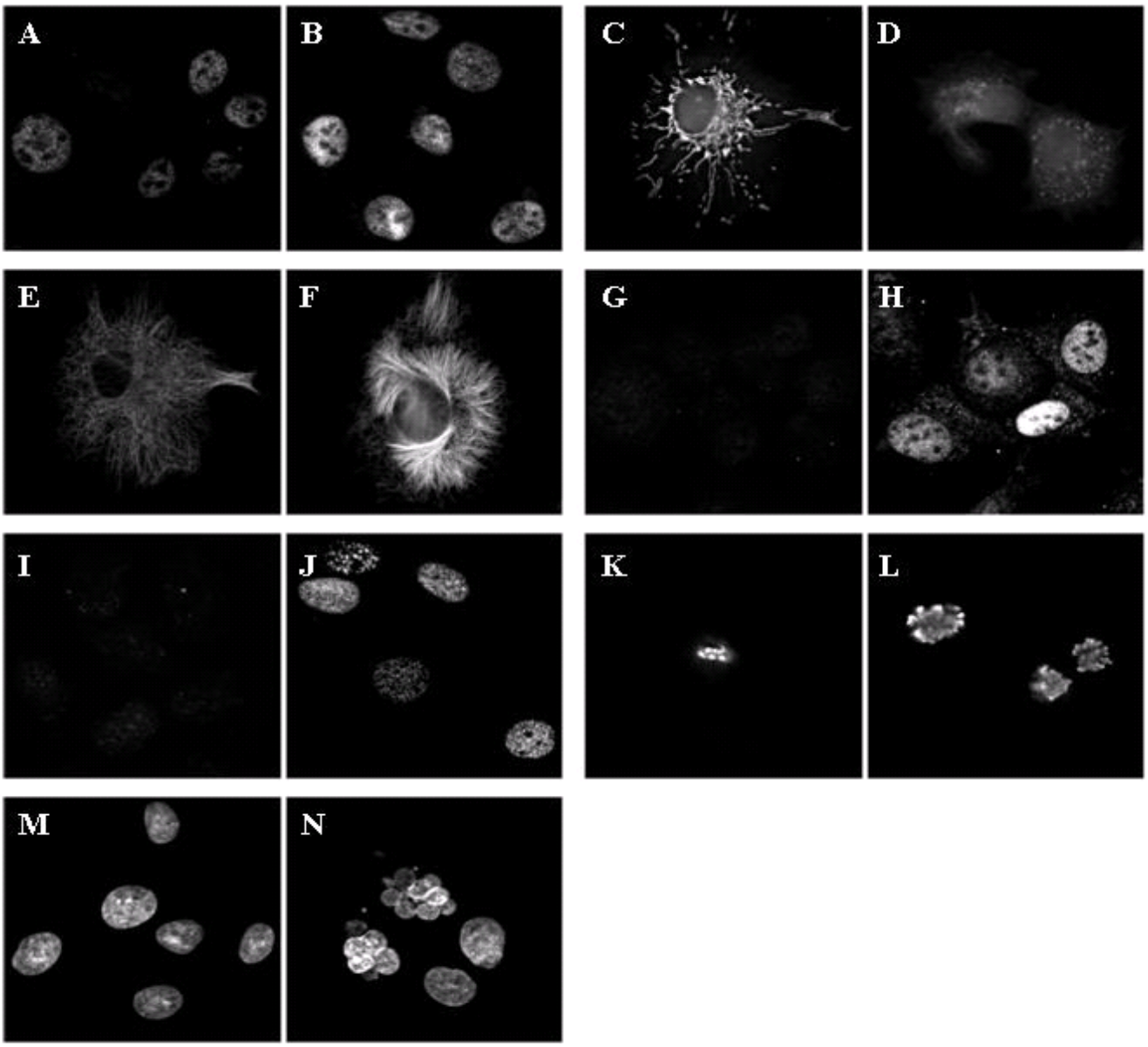
Cell Parameter	Parameter Details	Region of interest/ Measurement	Rationale
1. Cell Loss	Plates 1 and 2 Hoechst HCS Nuclear Stain; Paclitaxel positive control	Nuclear region. Count number of nuclei. Minimal nuclear clumping desirable.	Each nucleus represents 1 cell.
2. Cell Cycle Arrest	Plates 1 and 2 Hoechst HCS Nuclear Stain; Camptothecin positive control	Nuclear region. Use total intensity measurements to calculate ratio of number of 2n cells to number of 4n cells.	Increased nuclear intensity indicative of >2n nuclei.
3. DNA Degradation/ Apoptosis	Plates 1 and 2 Hoechst HCS Nuclear Stain; Paclitaxel positive control	Nuclear region. Use total intensity measurements to calculate % of cells with <2n nuclei. Can visualize apoptotic (fragmented) nuclei.	Decreased nuclear intensity indicative of <2n nuclei.
4. Nuclear Size	Plates 1 and 2 Hoechst HCS Nuclear Stain; Camptothecin positive control	Nuclear region. Calculate area of nuclear region.	Changes in nuclear size often associated with cytotoxicity.
5. Oxidative Stress	Plate 1 FITC-Phospho-Histone H2A.X; Anisomycin positive control	Nuclear region. Calculate average intensity over nucleus.	Increased probe intensity.
6. Stress Pathway Activation	Plate 1 Cy3-Phospho-c-jun; Anisomycin positive control	Nuclear region. Calculate average intensity over nucleus.	Increased probe intensity.

Cell Parameter	Parameter Details	Region of interest/ Measurement	Rationale
7. DNA Damage Response	Plate 1 Cy5-p53; Camptothecin positive control	Nuclear region. Calculate average intensity over nucleus.	Increased probe intensity.
8. Mitochondrial Membrane Potential	Plate 2 MitoDye; CCCP positive control	Cytoplasmic Ring. Calculate average mitochondrial intensity in the cytoplasmic ring.	Decreased probe intensity indicates decreased membrane potential.
9. Mitochondrial Mass	Plate 2 MitoDye; CCCP positive control	Cytoplasmic Ring. Calculate total mitochondrial area in cytoplasmic ring.	Increased probe area reflects increased mass.
10. Mitotic Arrest	Plate 2 FITC-Phospho-Histone H3; Paclitaxel positive control	Nuclear region. Calculate average intensity over nucleus.	Increased probe intensity.
11. Cytoskeletal Integrity	Plate 2 Cy5-α-tubulin; Paclitaxel positive control	Nuclear region. Calculate average intensity over nucleus.	Increased probe intensity indicates microtubule stabilization.

Data Handling

Cell feature information is extracted from images such as those shown in Figure 2 by the HCS processing algorithms, *e.g.,* software provided with the ArrayScan or IN Cell Analyzer (Table 8). Dose-response curves can be generated using curve fitting software (*e.g.,* GraphPad Prism) and tabulated as a feature set of log-scaled AC_{50} values (half-maximal active concentration). Expected AC_{50} values for the four control toxins are included in Table 9. Tabulated data can then be further assessed using a variety of informatics software such as SAS, S-PLUS, Spotfire or other similar software tools capable of generating hierarchical clustering, heat maps or other desired visual interfaces.

Sample Results



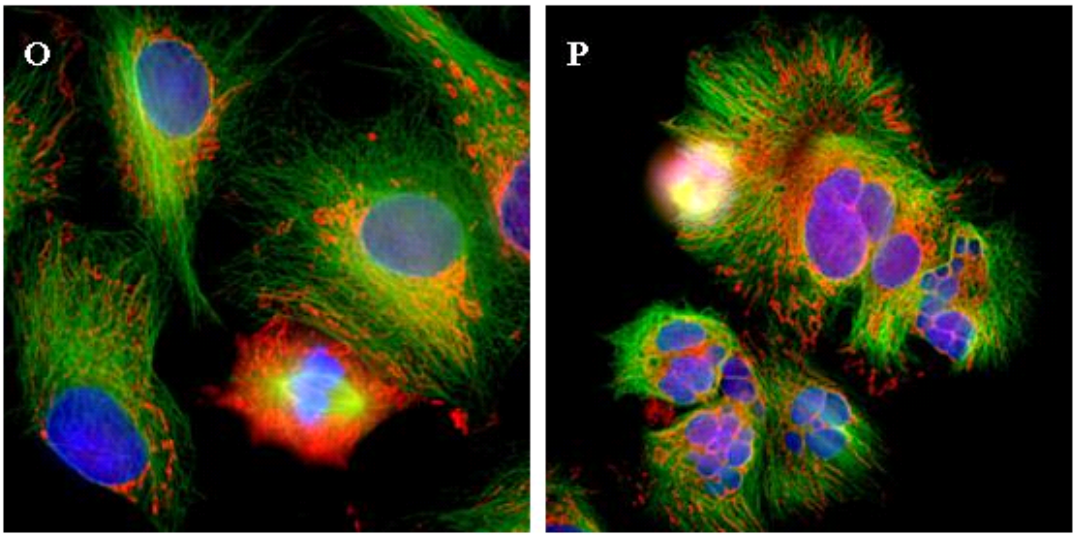


Figure 2. Image pairs of DMSO control and compound treated (24 hr) HepG2 cells.

Cell handling was as recommended in the HCS100 assay protocol; detection reagents and protocols used were as provided with the HCS100 assay kit. Images were obtained using a 40x/0.9 NA objective.

A&B. Cy3-c-jun control and anisomysin-treated cells. Untreated cells have some nuclear phospho-c-jun labeling (A) that becomes more intense with anisomycin treatment (B).

C&D. MitoDye control and CCCP-treated cells. Untreated cell has well defined mitochondria (C); cells treated with CCCP have less intense and more diffuse mitochondrial labeling (D).

E&F. Cy5-α-tubulin control and paclitaxel-treated cells. Untreated cell has an extensive array of single microtubules (E); cell treated with paclitaxel has stabilized microtubules with evidence of bundling (F).

G&H. Cy5-p53 control and camptothecin-treated cells. Untreated cells have low levels of p53 protein (G); cells treated with camptothecin exhibit activated nuclear p53 (H).

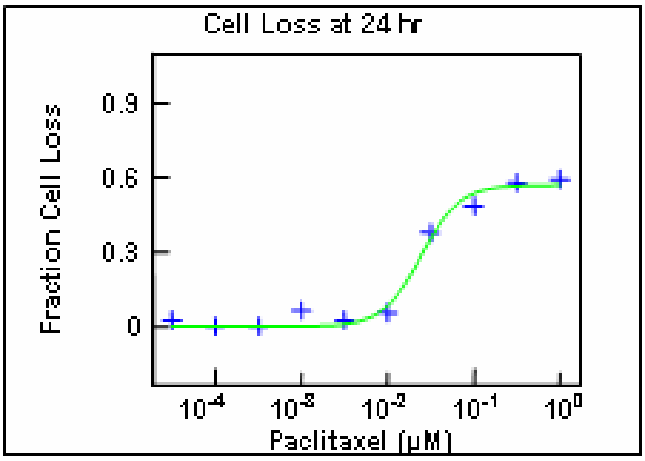
I&J. FITC-phospho-histone-H2A.X control and camptothecin-treated cells. Untreated cells maintain low levels of phospho-histone H2A.X (I); cells treated with camptothecin exhibit heterogeneously phosphorylated histone H2A.X within their nuclei (J).

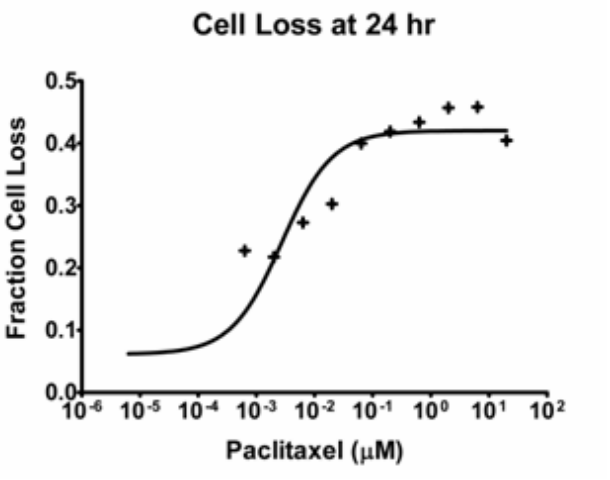
K&L. FITC-phospho-histone H3 control and paclitaxel-treated cells. Untreated cycling cells exhibit phospho-histone H3 only during normal mitoses (labeled metaphase chromosomes) (I); cells treated with paclitaxel show abnormal mitoses with elevated phospho-histone H3 (J).

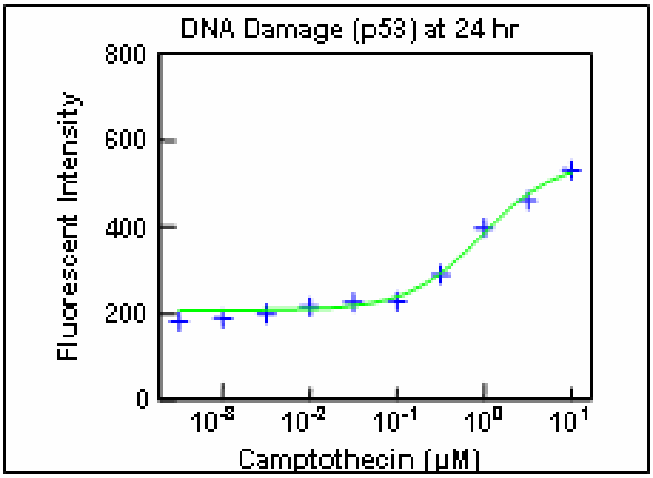
M&N. Hoechst HCS Nuclear Stain control and paclitaxel-treated cells. Untreated cells show normal nuclear morphology (M); subpopulations of cells treated with paclitaxel exhibit fragmented nuclei (N).

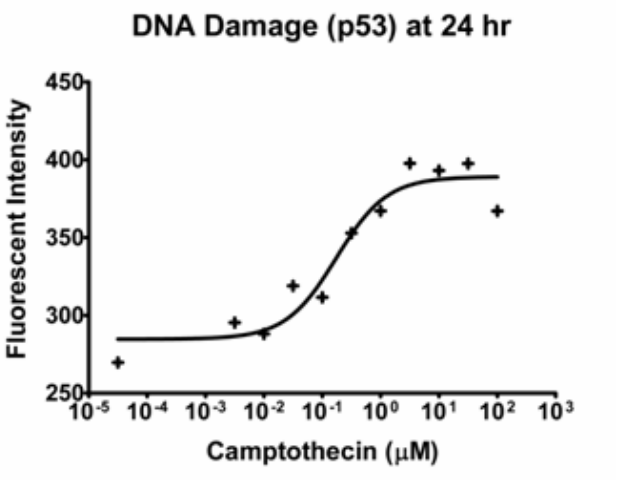
O&P. Four color pseudocolored image sets of control and paclitaxel-treated (24 hr) HepG2 cells. Blue – Nuclei; Green – Microtubules; Red – Mitochondria; Magenta – Phospho-Histone H3. Untreated cells show a well-defined microtubule cytoskeleton and normal mitochondria and nuclei. The image also includes one cell undergoing mitosis (metaphase) (O). When treated with paclitaxel, subpopulations of cells exhibit bundled microtubles, fragmented nuclei, and elevated phospho-histone H3 levels (P).

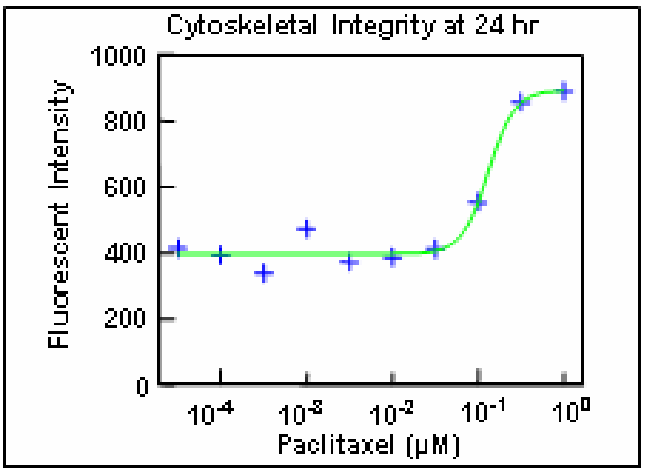
Figure 3. Examples of paclitaxel, anisomycin and camptothecin curve fit dose-response relationships at 24 hr.

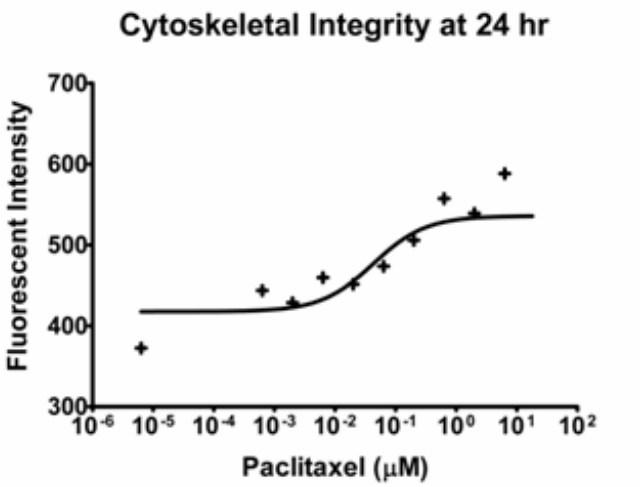












Left Panel. Data obtained from plates imaged and analyzed using the ArrayScan VTI (ThermoFisher), with plots generated from ExcelFit software.

Right Panel. Data obtained from plates imaged and analyzed using the GE IN Cell Analyzer 1000, with plots generated from GraphPad Prism software.

Table 9. Typical AC_{50} values of selected cell features

Cell Feature	AC_{50}	Time point	Compound
Cell Loss (Hoechst HCS Nuclear Stain)	10 nM	72 hr	Paclitaxel
Cell Cycle Arrest (Hoechst HCS Nuclear Stain)	96 nM	24 hr	Camptothecin
Nuclear Size (Hoechst HCS Nuclear Stain)	107 nM	24 hr	Camptothecin
Oxidative Stress (Phospho-Histone H2A.X)	20 nM	1 hr	Anisomycin
Stress Pathway Activation (Phospho-c-jun)	21 nM	1 hr	Anisomycin
DNA Damage Response (p53)	1.8 µM	24 hr	Camptothecin
Mitochondrial Membrane Potential (MitoDye)	19.7 µM	1 hr	CCCP
Mitochondrial Mass (MitoDye)	3.4 µM	1 hr	CCCP
Mitotic Arrest (Phospho-Histone H3)	188 nM	24 hr	Paclitaxel
Cytoskeletal Integrity (α-Tubulin)	600 nM	24 hr	Paclitaxel

Troubleshooting Guide

Problem	Potential Explanations/Solutions
Cell loss	Optimize liquid aspiration/ dispensation rate to reduce cell loss due to shear. Optimize extracellular matrix protein-coating to improve cell adhesion to microplate. Cell loss due to toxic treatments may hinder statistically relevant analysis; alter toxin dosages/treatment times to reduce cell loss levels. Pre-warm fixation solution to 37ºC prior to addition.
Low signal	Improper storage or preparation of primary/secondary antibodies or dyes – retry stain with fresh antibody/dye solution. If utilizing alternate cell type, inadequate primary/secondary antibody or dye concentrations for cell type – titrate dilutions to optimize signal. Signal may diminish in extremely dense cultures – decrease cell seeding concentration or increase concentrations of primary/secondary antibodies or dyes. Antibody incubation times may vary with alternate plate formats (*e.g.*, 1536-well); longer incubation times may be necessary. Optimize exposure times and/or fluorescence filters appropriate to fluorophore. Insufficient numerical aperture of objective lens – increase if possible.
High background	Improper reagent storage or preparation – retry with fresh reagent (antibodies/dyes or buffers). Samples may have dried during staining – retry stain on fresh samples. If utilizing alternate cell type, excessive primary/secondary antibody or dye concentrations for cell type – titrate dilutions to optimize signal. Additional wash steps after the primary/secondary antibody incubations may help decrease background fluorescence due to non-specific binding. Check for autofluorescence of microplate.

Problem	Potential Explanations/Solutions
Poor focus/out-of-plane blurring	Imaging at high magnifications with shallow depths of field may cause focusing difficulties – use lower magnification. Plate irregularities – perform autofocusing/auto-offset at each well location or use plates with more consistent dimensions (*e.g.*, well-bottom flatness, thickness)
Cannot obtain measurement parameter from HCS platform software	Check image focusing, optimize exposure times and fluorescence filters, and verify imaging algorithm configuration using responses from positive control compounds. Verify accuracy of nuclear/cytoplasmic segmentation. Effective segmentation parameters can be HCS system/ software dependent. Consider increasing cell trituration or decreasing cell seeding concentrations/durations of culture for difficulty in analysis of dense cultures (separation of multiple nuclei/cell bodies).
Poor reproducibility; cannot generate positive control dose response curves.	Insufficient imaging quantity – increase number of cells analyzed for condition. Efficacy of control or test 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 parameters of interest. Perform time-course experiments to determine kinetics of compound effects for cell type/parameter of interest. Shorter/longer treatment durations may be required.

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