High Content Screening Assay for Hepatotoxicity using Human HepG2 cells

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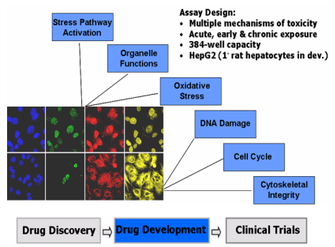
Abstract

Drug-induced hepatotoxicity is a major factor in both the high fail rate of drug development and withdrawal of drugs from the market. Consequently, there is a need for more powerful test systems to identify potential hepatotoxins. High Content Screening enables the investigation of cells as integrated and interacting networks of genes, proteins and metabolic processes that give rise to either normal or pathological conditions. Though multiplexing of high specificity detection reagents, High Content Screening assays can extract a systemic cellular response to a treatment or condition in the form of a panel of features measured in specific cell types. Millipore has developed a High Content Screening assay for hepatotoxicity using human HepG2 cells. The assay comprises detection reagents and protocols for profiling up to eleven hepatotoxicity endpoints, including Cell Loss, Cell Cycle Arrest, DNA Degradation/Apoptosis, Nuclear Size, Oxidative Stress, Stress Kinase Activation, DNA Damage, Mitochondrial Membrane Potential, Mitochondrial Mass, Mitotic Arrest and Cytoskeletal Integrity. The commercially available assay kit allows analysis of all parameters at 3 time-points for 4 control toxins and up to 16 unknown compounds in two 10-point dose response curves. We present data showing that the reagents and protocols are suitable for analysis on widely-used HCS readers, such as the GE IN Cell Analyzer 1000 and ThermoFisher ArrayScan. Our data demonstrate the kit's effectiveness in the detection of each parameter's response to control toxins. The combination of unique antibody pairs and detection reagents allows comprehensive assessment and analysis of the cellular response to hepatotoxin challenge. The assay may be used to filter compounds for toxicity and thus has utility for compound ranking for efficacy or pre-clinical safety assessment.

Introduction

High-Content Screening (HCS) technology represents a major step towards improving the drug discovery process. HCS enables the evaluation of multiple biochemical and morphological parameters in cellular systems. High Content Screening is the next dimension in cell analysis using imaging technologies. Recent data indicate that multiparametric High-Content Screening for hepatotoxicity using human HepG2 cells has a high concordance with drug-induced human hepatotoxicity (O'Brien et al., 2006). Millipore have developed a Hepatotoxicity Profiling Assay for human HepG2 cells, a multiplexed HCS kit comprising high-quality, validated, compatible detection reagents and validated protocols for profiling 11 human hepatotoxicity end-points in two 384 well microplates. This Hepatotoxicity Profiling Panel for human HepG2 cells 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. The assay allows detection of 11 parameters at three separate time points (e.g., acute – 30 min, 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.

Assay Design



Screen compounds for a broad range of potentially toxic effects early in the drug development process

Accelerate prioritization of lead compounds

Gain insights into mechanism(s) underlying drug actions

Protocol Overview

1. Plate HepG2 cells on 384 well microplates. Incubate for 18 hr.
2. Treat cells with control toxins (below) 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
3. Label cells with physiological indicator dyes, primary antibodies and corresponding secondary antibodies.
4. Perform HCS imaging and analysis.
5. Data Interpretation - obtain Human Cytotoxicity Profile

Plate Layouts

Multiplex Plate 1		
Cell Parameter	**Measurement**	**Detection**
1. Cell loss	Cell number	Nuclear Stain
2. Cell cycle arrest	DNA content	Nuclear Stain
3. DNA Degradation	DNA structure	Nuclear Stain
4. Nuclear size	Nuclear area	Nuclear Stain
5. Oxidative Stress	Phosphorylation	Phospho-Histone H2A.X
6. Stress Pathway Activation	Phosphorylation	Phospho-c-jun
7. DNA Damage Response	Target activation	p53

Multiplex Plate 2		
Cell Parameter	**Measurement**	**Detection**
1. Cell loss	Cell number	Nuclear Stain
2. Cell cycle arrest	DNA content	Nuclear Stain
3. DNA Degradation	DNA structure	Nuclear Stain
4. Nuclear size	Nuclear area	Nuclear Stain
8. Mitochondrial function I	Mitochondrial membrane potential	MitoDye
9. Mitochondrial function II	Mitochondrial Mass	MitoDye
10. Mitotic Arrest	Phosphorylation	Phospho-Histone H3
11. Cytoskeletal Integrity	Microtubule Stability	α-Tubulin

Tables 1& 2. Antibody Selection and Cellular Response. Cytotox Profile: Multiplex Plates 1 & 2

Results

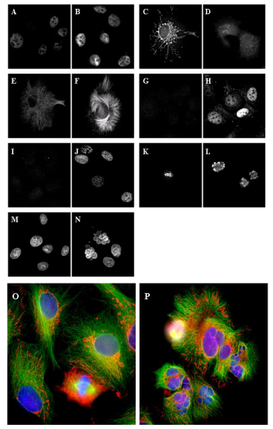


Figure 1. Image pairs of DMSO control and compound treated (24 hr) HepG2 cells. A&B. Effect of anisomycin on stress pathway (c-jun) activation. Untreated cells have some nuclear labeling (A) that becomes more intense with 40 µM anisomycin treatment (B). **C&D.** Effect of CCCP on mitochondrial function. Untreated cell has well defined mitochondria (C), cells treated with 126 µM CCCP have less intense, more diffuse mitochondrial labeling (D). **E&F**. Effect of paclitaxel on cytoskeletal integrity/microtubule stability. Untreated cell has an extensive array of single microtubules (E), cell treated with 20 µM paclitaxel has stabilized microtubules with evidence of bundling (F). **G&H.** Effect of camptothecin on DNA damage (p53) response. Untreated cells have low levels of p53 (G), cells treated with 32 µM camptothecin exhibit activated p53 (H). **I&J**. Effect of camptothecin on oxidative stress. Untreated cells maintain low levels of oxidative stress marker Phospho-Histone H2A.X (I), cells treated with 100 µM camptothecin exhibit elevated levels of Phospho-Histone H2A.X within their nuclei (J). **K&L.** Effect of paclitaxel on mitotic arrest. Untreated cycling cells exhibit mitotic arrest marker Phospho-Histone H3 only during normal mitoses (I), cells treated with 6.3 µM paclitaxel show abnormal mitoses with elevated Phospho-Histone H3 (J). **M&N**. DNA degradation marker and 20 µM paclitaxel. Untreated cells show normal nuclear morphology (M), subpopulations of cells treated with 20 µM paclitaxel exhibit fragmented nuclei (N). **O&P**. Image sets of control and 0.2 µM 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 (O). When treated with 0.2 µM paclitaxel, subpopulations of cells exhibit bundled microtubules, fragmented nuclei, and elevated Phospho-Histone H3 levels (P).

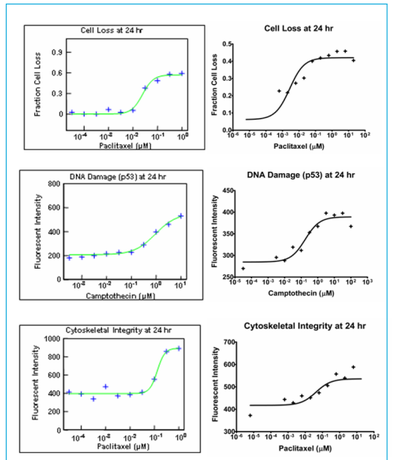


Figure 3. Examples of cytotoxicity profiles generated using the hepatotoxicity Assay, and demonstration of assay platform-independence. Data demonstrating the effects of paclitaxel or camptothecin upon cell loss, DNA damage and microtubule stability. Left Panels represent data obtained from plates imaged and analyzed using Cellomics' ArrayScan VTI. Right Panels represent data obtained from plates imaged and analyzed using GE's IN Cell Analyzer 1000, with plots generated from GraphPad Prism software. Cell handling was performed as recommended in the HCS100 assay protocol; detection reagents and protocols used were as provided with the HCS100 assay kit.

Cell Feature	AC_{50}	Time point	Compound
Cell Loss	10 nM	72 hr	Paclitaxel
Cell Cycle Arrest	96 nM	24 hr	Camptothecin
Nuclear Size	107 nM	24 hr	Camptothecin
Oxidative Stress	20 nM	30 min	Anisomycin
Stress Pathway Activation	21 nM	30 min	Anisomycin
DNA Damage Response	1.8 µM	24 hr	Camptothecin
Mitochondrial Mem. Pot.	19.7 µM	30 min	CCCP
Mitochondrial Mass	3.4 µM	30 min	CCCP
Mitotic Arrest	188 nM	24 hr	Paclitaxel
Cytoskeletal Integrity	600 nM	24 hr	Paclitaxel

Table 3. Data analysis shows a direct correlation to known cytotoxic agents. Using the assay, AC_{50} values of a range of known toxic compounds upon multiple parameters were generated. The three time points used in this assay (30 min, 24 hr or 72 hr exposure) allow the end-user to distinguish between acute and chronic drug actions.

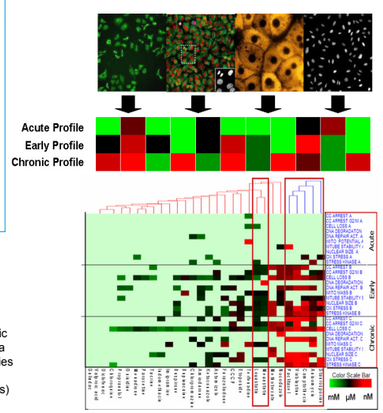


Figure 4. Data Analysis. The assay starts with a set of cellular biomarkers selected to capture the cellular systemic response to toxic challenge. Image analysis for a panel of cellular functions produces a systems response profile. Clustering of compound responses identifies profiles that correlate with cellular functions, allowing prioritization of lead compounds and providing an understanding of the mechanism(s) of drug action.

Kit Components

- Antibody A, 100x. Anti-phospho-histone H2A.X: 1 vial containing 100 µL
- Antibody B, 200x. Anti-Antibody A, FITC conjugate: 1 vial containing 100 µL
- Antibody C, 100x. Anti-phospho-c-jun: 1 vial containing 100 µL
- Antibody D, 200x. Anti-Antibody C, Cy3 conjugate: 1 vial containing 100 µL
- Antibody E, 100x. Anti-p53: 1 vial containing 100 µL
- Antibody F, 200x. Anti-Antibody E, Cy5 conjugate: 1 vial containing 100 µL
- Antibody G, 100x. Anti-phospho-histone H3: 1 vial containing 100 µL
- Antibody H, 200x. Anti-Antibody G, FITC conjugate: 1 vial containing 100 µL
- Antibody I, 100x. Anti-α-tubulin: 1 vial containing 100 µL
- Antibody J, 200x. Anti-Antibody I, Cy5 conjugate: 1 vial containing 100 µL
- MitoDye Solution, 10,000x: 1 vial containing 15 µL
- Nuclear Stain Solution, 200x: 1 vial containing 200 µL
- Compound Dilution Buffer: 1 bottle containing 500 mL
- Cell Fixation Buffer: 1 bottle containing 200 mL
- Cell Permeabilization Buffer: 1 bottle containing 100 mL
- Antibody Dilution Buffer: 1 bottle containing 100 mL
- Wash Buffer: 2 bottles each containing 1000 mL
- Control Toxin 1, Anisomycin, 2.5 mM in DMSO, 250x: 1 vial containing 100 µL
- Control Toxin 2, Camptothecin, 2.5 mM in DMSO, 250x:1 vial containing 100 µL
- Control Toxin 3, CCCP, 25 mM in DMSO, 250x: 1 vial containing 100 µL
- Control Toxin 4, Paclitaxel, 0.25 mM in DMSO, 250x: 1 vial containing 100 µL
- 20 gauge needle with syringe, 5cc capacity: 6 each, sterile, disposable
- Plate Sealers: 6 each

Summary and Conclusions

- Screen compounds for a broad range of potentially toxic effects early in the drug development process.
- Accelerate prioritization of lead compounds.
- Gain insights into mechanism(s) underlying drug actions.
- Relevant to human cytotoxicities.
- Proprietary panel of high quality detection reagents provides high sensitivity and selectivity.
- Multiplexed analysis of eleven human cytotoxicity parameters using two 384 well microplates.
- Detection of eleven human cytotoxicity parameters over up to 3 time points.
- Generate ten-point dose response curves in duplicate for up to 16 test compounds and 4 control toxins (provided).
- Optimized HepG2 cell handling protocol enables consistent cell plating.
- Fully leverages High-Content Screening.

References

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