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WuXi AppTec

WuXi Biology
Enabling Unbounded Possibilities

WuXi AppTec
In Vitro Toxicity Services

Identify potential liability issues in early stages of drug discovery

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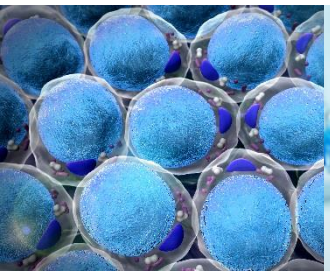



Introduction to *In Vitro* Toxicity

Drug induced toxicity is one of the leading causes of drug candidate failure in preclinical and clinical testing stage, and also the major reason for the withdrawal of approved drugs from the market. Initial toxicity testing is required during the nonclinical phase of development and relies primarily on animal studies. While these animal models have provided useful information on the safety of chemicals, they are relatively expensive, low-throughput, and sometimes inconsistently predictive of human biology and pathophysiology because of the species difference. With increasing numbers of new chemical entities for environmental and pharmaceutical uses, it is necessary to find a rapid and efficient method to screen chemicals for their potential toxicities. Since most drug-induced toxicity is due to toxic effects at the cellular level, alternative *in vitro* models are increasingly being used to estimate *in vivo* responses, to reduce and/or replace *in vivo* animal testing, and to increase the throughput. This idea is supported by the 2007 NRC report, "Toxicity Testing in the 21st Century (TT21C): A Vision and a Strategy[1]." This report predicts substantial advances in toxicity testing in the near future which are much more specific and predictive of human toxicity. *In vitro* toxicity testing studies are faster, simpler and more scalable so they can be used in the early drug discovery stage to predict potential risk. This would not only be economical but ethical as it could markedly reduce the number of animal usage.

To address the need for early stage *in vitro* toxicity testing, WuXi AppTec Biology offers a panel of toxicity assays at the cellular level by utilizing cutting-edge technologies, such as conventional and automated patch clamp and high content screening (HCS). Applying these assays to your lead ID and optimization strategy can help provide a more thorough analysis of the severity and specificity of toxicity. This information can then be used to guide candidate compounds through the planning and execution of downstream *in vivo* tests.

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Cardiotoxicity

Unintended drug-induced arrhythmia, in particular Torsade de Pointe arrhythmia (TdP), have been responsible for approximately 21.4% of drug withdrawals from markets between 1990 and 2012. For the past decade, cardiac safety screening studies have been conducted according to ICH S7B and ICH E14 guidelines. The ICH S7B guideline includes an *in vitro* IKr (hERG) assay and an *in vivo* ECG assay to identify the potential for delayed repolarization (QT interval prolongation). Although hERG is the most important channel related to the risk of TdP, hERG screening alone cannot reliably detect potential cardiac adverse side effects. Furthermore, this over-simplified and highly sensitive approach can result in unwarranted attrition of novel drug candidates owing to false-positive findings. Recently a new paradigm to examine cardiotoxicity called the Comprehensive *in Vitro* Proarrhythmia Assay (CiPA) was proposed to replace the current ICH S7B/E14 guidelines. The CIPA core assays include: 1. the assessment of drug candidate effects on multiple human ventricular ionic channels and in silico reconstruction of human heart ventricular action potential to predict the proarrhythmic risk; and 2. the confirmation study using human stem-cell derived cardiomyocytes[2,3].

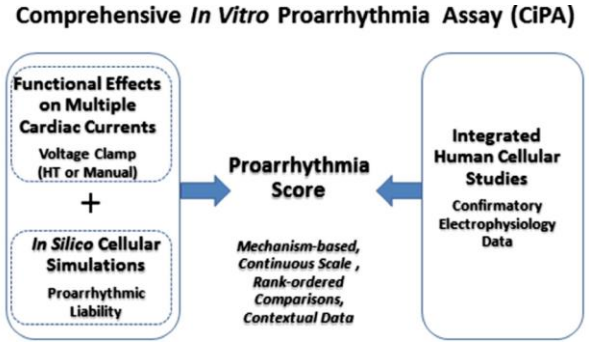


Figure 1. Diagrammatic representation of CiPA[2]

Ion Channel	Species	Assay platform
hERG (I_{Kr})	human	Automated or Manual Patch Clamp
Cavl.2 (I_{Ca}, L-type)	human	Manual Patch Clamp
Navl.5 (I_{Na} - peak current)	human	Automated or Manual Patch Clamp
Navl.5 (I_{Na} - late current)	human	Automated or Manual Patch Clamp
Kv4.3/KChip2.2 (I_{TO})	human	Automated or Manual Patch Clamp
KCNQ1/mink (I_{Ks})	human	Manual Patch Clamp
Kir2.1 (I_{K1})	human	Automated or Manual Patch Clamp

Table 1. Cardiac ion channel panel (CIPA recommended[3]).

WuXi AppTec provides a combination of assay platforms that include manual and automated patch-clamp and microelectrode array (MEA) to access drug candidate effects on multiple cardiac ion channels (Table 1) as well as stem cell-derived ventricular cardiomyocytes as CIPA recommended.

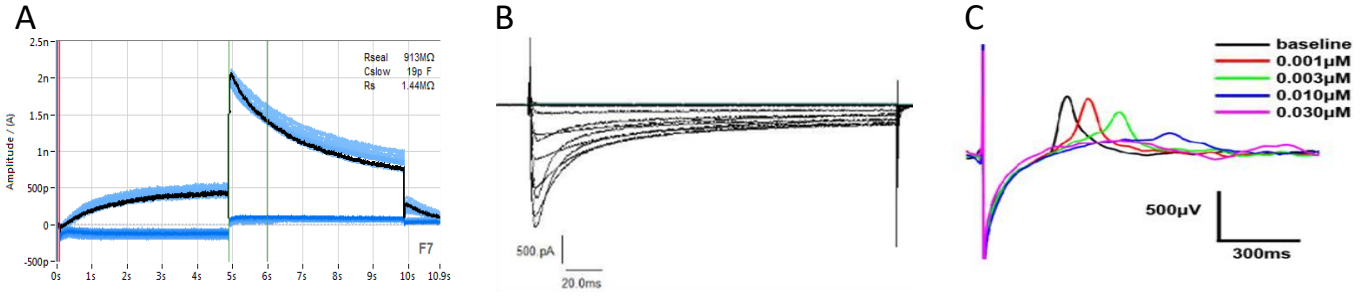


Figure 2. Sample traces recorded from SyncroPatch automatic patch clamp system (A), Manual patch clamp (B) and MEA system (C). The cell line used in SyncroPatch is the CHO-hERG stable cell line, while in Manual patch clamp is HEK-Cav1.2 stable cell line. hiPSC-vCMs were used in the MEA system and the effects of dofetilide were recorded.

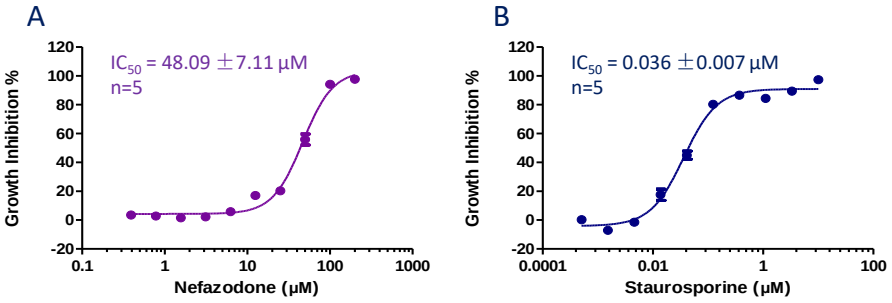
WuXi AppTec Biology collaborates with a third party vendor for the human induced pluripotent stem cell-derived ventricle cardiomyocytes (hiPSC-vCMs). The purity of the ventricular cardiomyocytes is over 90%, which is the highest number seen in the literature. The action potential, main ion channel properties and development process of the hiPSC-vCMs have all been fully validated using the manual patch clamp system at WuXi AppTec[4,5]. WuXi AppTec Biology now offers CIPA confirmatory electrophysiological test[3] using MEA (Maestro Pro, Axion) to analyze the effects of compounds on hiPSC-vCMs.

In addition, to satisfy the mechanistic study or high throughput study needs, WuXi AppTec Biology also provides traditional radioligand binding and fluorescence signal detection assays. The service for some other cardiac targets such as Kv1.5 (Ikur) and Cav3.2 (ICa,T) is also available.

General Cytotoxicity

Cytotoxicity testing is mandated by the FDA and CFDA for IND/CTA submission and is typically performed during the nonclinical phase of discovery. In recent decades, it is well accepted that *in vitro* cytotoxicity testing methods should be considered before animals are dosed to examine acute oral systemic toxicity. Identifying potential cytotoxicity early in the process can dramatically save both time and capital by eliminating likely toxic compounds prior to pivotal animal studies. Additionally cell-based assays are easy to scale for medium or high throughput screening and guiding go/no-go decisions.

● Cell Viability: ATP production measured in active cells



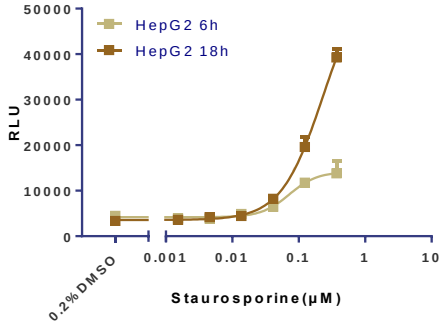
(A) In a 384-well HepG2 cell viability assay, 24 hours of Nefazodone treatment inhibited cell viability in a concentration dependent manner (n=5).

(B) After 72 hours treatment in 384-well plates, Staurosporine showed concentration dependent inhibition on the viability of HepG2 cells (n=5).

The CellTiter-Glo® Luminescent Cell Viability Assay (Promega): This is a homogeneous method to determine the number of viable cells based on quantitation of the ATP present, which is a marker for the presence of metabolically active cells. WuXi AppTec Biology has fully validated this assay with a wide range of cell types, in both 96- and 384-well format, using EnVision (Perkin Elmer) as the luminescent signal reader.

● Apoptosis: Caspase assay

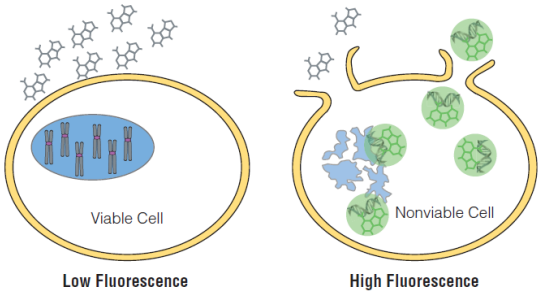
Activation of the caspase cascade is an integral event in the apoptotic pathway. WuXi AppTec Biology uses Caspase-Glo® 3/7 Assay kit (Promega) to measure caspase-3 and caspase-7 activities. The assay was fully validated on HepG2 Cells with EnVision as the Luminescence signal reader. Available in both 96- and 384-well format.



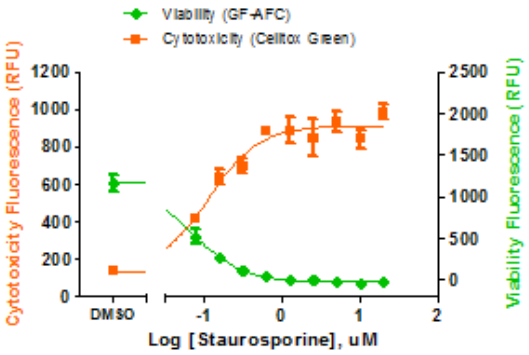
Staurosporine increased caspase activity. In a 96-well plate assay staurosporine concentration-dependently increased caspase activities in the HepG2 cells. EC_{50} was 0.07 μM and 0.23 μM for 6-hour and 18-hour staurosporine treatment, respectively.

● CellTox™ Green Cytotoxicity Assay

The CellTox™ Green Cytotoxicity Assay (Promega) measures changes in membrane integrity that occur as a result of cell death. The assay system uses a proprietary asymmetric cyanine dye that is excluded from viable cells but preferentially stains dead cell DNA. Viable cells produce no appreciable increases in fluorescence. Therefore, the fluorescent signal produced by the dye binding to the dead-cell DNA is proportional to cytotoxicity. We now offer this assay in both 96- and 384-well format on various cell types, using Envision as the signal detector. This assay can be combined with CellTiter-Fluor™ Cell Viability Assay or CellTiter-Glo® Luminescent Cell Viability Assay.



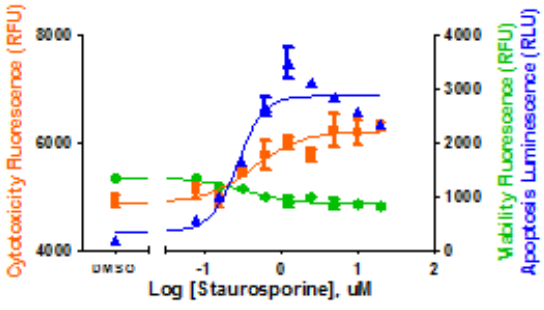
CellTox™ Green Dye binds DNA of cells with impaired membrane integrity.



LN-18 cells were exposed to staurosporine with indicated concentrations in a 384-well assay plate for 24 hours. Fluorescence associated with cytotoxicity was measured after CellTox™ Green Reagent was applied, then the CellTiter-Fluor Reagent was applied and fluorescence signal was measured. These measurements produced similar EC_{50} values.

● ApoTox-Glo™ Triplex Assay (Promega)

The ApoTox-Glo™ Triplex Assay (Promega) combines three Promega assay chemistries to assess viability, cytotoxicity and caspase activation events within a single assay well. The first part of the assay simultaneously measures two protease activities; one is a marker of cell viability, and the other is a marker of cytotoxicity. The second part of the assay uses the Caspase-Glo® Assay Technology to detect caspase activities. WuXi AppTec has validated this assay with various cell types, using Envision as the signal reader.



In the 384-well assay with LN-18 cells, seven hours staurosporine treatment caused concentration-dependent decrease in cell viability, increase in cytotoxicity and increase in caspase-3/7 activities.

Mitochondrial Toxicity

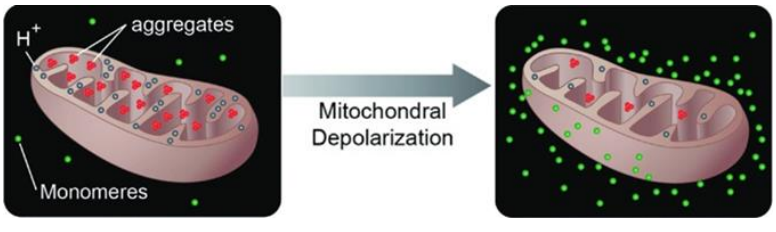
Mitochondria play a pivotal role in cellular energy (ATP) production and maintaining homeostasis. Mitochondrial dysfunction is increasingly implicated as a major contributor to drug-induced toxicity, leading to the discontinuation of prominent drugs, including troglitazone, cerivastatin and nefazodone. In addition to post-market drug withdrawals, mitochondrial liabilities have also been associated with many drugs carrying a black box label for hepatic and cardiac toxicity.

WuXi AppTec offers a set of *in vitro* assays to access mitochondrial toxicity from different endpoint.

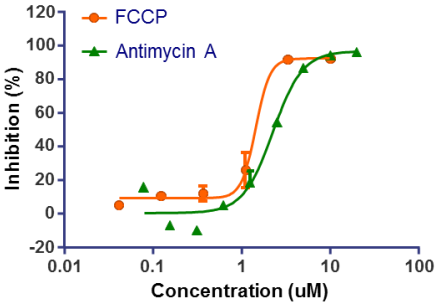
● Mitochondrial Membrane Potential Assay

Mitochondrial membrane potential (MMP) is tightly interlinked to many mitochondrial processes so it is a key indicator of mitochondrial function and cell health. The dissipation of MMP is considered an early indicator of apoptosis.

WuXi Biology offers a plate based HCS assay to detect the MMP, using the Acumen Cellista (TTP Labtech) with MITO-ID® Membrane Potential Cytotoxicity Kit (ENZO Life Sciences). The assay is available in both 96- or 384-well format, and in a wide range of cells.



The MITO-ID® Membrane Potential Cytotoxicity Kit utilizes a cationic dual-emission dye that exists as green fluorescent monomers in the cytosol, and accumulates as orange fluorescent aggregates in the mitochondria. Cells exhibit a shift from orange to green fluorescence as mitochondrial function becomes increasingly compromised.



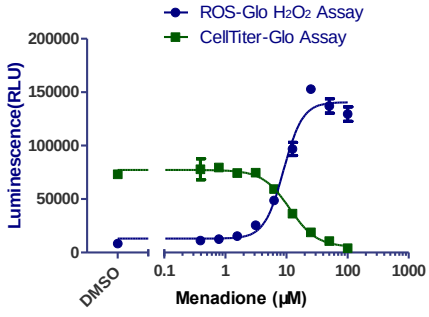
Compound	IC_{50} (μM)	Literature (μM)[6]
FCCP	1.12 ± 0.55	1.31
Antimycin A	2.56 ± 0.63	4.51

After one hour treatment, two references compounds, FCCP and Antimycin A, showed MMP inhibition in the 384-well assay plate using HepG2 cells (n=4). The IC_{50} values are close to the literature.

● Mitochondrial Reactive Oxygen Species (ROS) Assay

Mitochondrial dysfunction usually causes increased free radical production. The predominant source of free radical generation is the mitochondrial respiratory chain, and inhibition of this process is often connected to increased levels of reactive oxygen species (ROS). In the different ROS generated in cell culture, H_2O_2 is convenient to assay because of the long half-life in cultured cells. A change in H_2O_2 can reflect a general change in the ROS level.

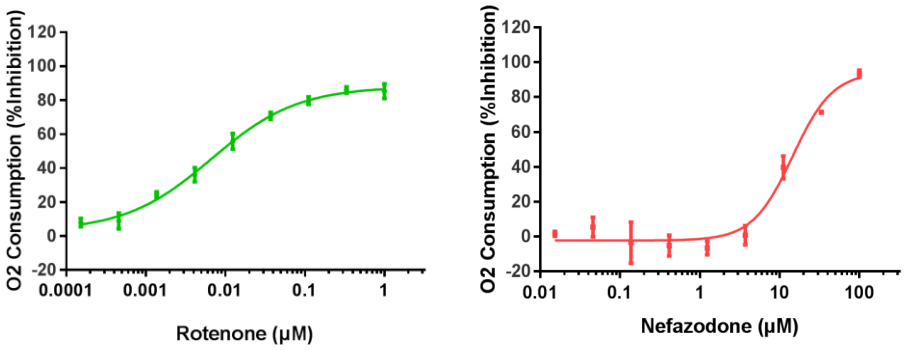
WuXi Biology has validated a plate based assay using ROS-Glo™ H_2O_2 assay kit (Promega), or Amplex® Red Hydrogen Peroxide/Peroxidase Assay Kit (Invitrogen), to detect H_2O_2 levels. The assay is available in both 96- or 384-well format.



In this representative example, the ROS-Glo™ H_2O_2 assay and the CellTiter-Glo® Luminescent Cell Viability assay were performed on the same HepG2 cells in the 384-well format. The cells were treated with ROS-generating compound menadione as well as H_2O_2 substrate. After incubation at 37°C for two hours, the half volume of supernatant was used for ROS-Glo™ H_2O_2 detection, while the cells were lysed for CellTiter-Glo® detection. The luminescence signal from both assays measured with EnVision. Menadione had an EC_{50} of 8.96 μM in the H_2O_2 assay, and an IC_{50} of 11.28 μM in the viability assay.

● Oxygen Consumption Assay: MitoXpress® Xtra OCR Assay

The MitoXpress® Xtra assay (Luxcel) directly measures immediate and acute drug effects on mitochondrial oxidative phosphorylation (Oxygen Consumption Rate, OCR) and is the most effective high throughput screen for Mitochondrial Toxicity using whole cells. Oxygen consumption is the most important parameter for the direct and specific assessment of the function of the electron transport chain, the cornerstone of oxidative phosphorylation and cellular metabolism. In the MitoXpress® Xtra OCR assay, the MitoXpress® reagent is quenched by oxygen, whereby oxygen depletion caused by mitochondrial activity causes an increase in probe signal, with rates of oxygen consumption calculated from the changes in fluorescence signal over time. Available in a 384- or 96-well high throughput format, this assay is compatible with a very wide range of primary, iPS or cell line models and both 2D and 3D culture systems.

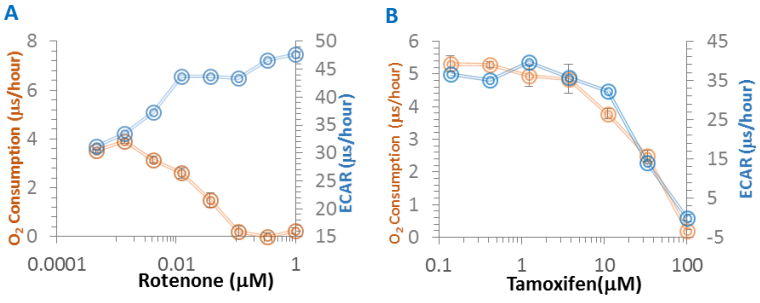


Compound	IC_{50} (μM)	Literature (μM)[7]
Rotenone	0.029 ± 0.007	0.020 ± 0.007
Nefazodone	13.90 ± 0.88	12.65 ± 2.43

Results from a typical 96-well MitoXpress® Xtra OCR concentration-response assay for two mitochondrial inhibitors, Rotenone and Nefazodone. The HL60 cells were used in the assay.

MitoXpress® Cellular Energy Flux OCR/ECAR Assay

A deeper, investigative analysis to understand the mechanism of mitochondrial toxicity and its relationship to cellular ATP production is made possible through the addition of Luxcel Biosciences pH Xtra – Glycolysis Assay; detecting in real-time the combined effects on OCR and extracellular acidification rate (ECAR)[5]. This assay is available in a 384- or 96-well high throughput format.



Results from typical 384-well MitoXpress® Cellular Energy Flux OCR/ECAR assay with HL60 cells, illustrating the difference between a classic mitochondrial inhibitor, Rotenone, which decreased OCR / increased ECAR (A); and a non-specific cytotoxic drug, Tamoxifen, which decreased both OCR and ECAR (B).

Glucose/Galactose Assay

Replacing glucose with galactose in the cell media increases the reliance of the cells on mitochondrial oxidative phosphorylation, thereby increasing susceptibility to the implications of mitochondrial insult. By comparing the differential toxic effects on glucose and galactose grown cells it will differentiate mitochondrial toxicity from non-specific cytotoxicity.

We use HepG2 cells cultured in either glucose (25 mM) or galactose (10 mM), with cytotoxicity assessed using CellTitre-Glo™ (Promega). A mitochondrial toxicant is indicated by a greater than three-fold change in IC_{50} value observed in the glucose media compared to the galactose media.

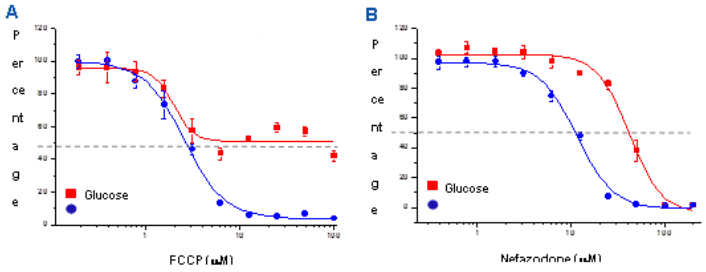


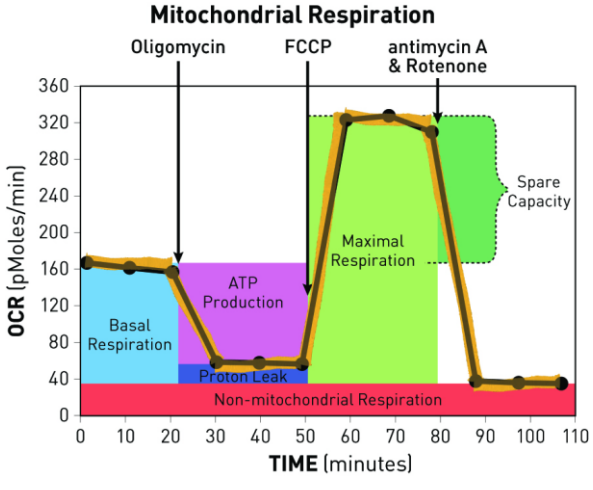
Illustration of the 24-hour treatment data for the mitochondrial uncoupler, FCCP (A), and inhibitor, nefazodone (B). A > 3-fold and 3.7-fold increase in IC_{50} value is observed for FCCP and nefazodone, respectively, in glucose media compared with galactose media.

Seahorse Cellular Mitochondrial Stress Assay

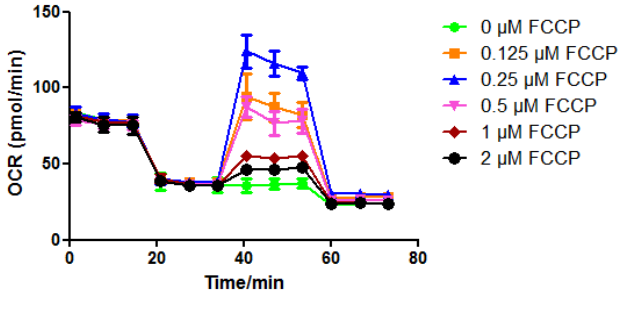
The Agilent Seahorse XF Cell Mito Stress Test measures cellular metabolism via the rate of change in oxygen concentration and pH, to calculate the Oxygen Consumption Rate (OCR, the indicator of mitochondrial respiration) and Extracellular Acidification Rate (RCAR, the indicator of glycolysis).

Sequential compound injections measure basal respiration, ATP production, proton leak, maximal respiration, spare respiratory capacity, and nonmitochondrial respiration.

XFe96 Analyzer



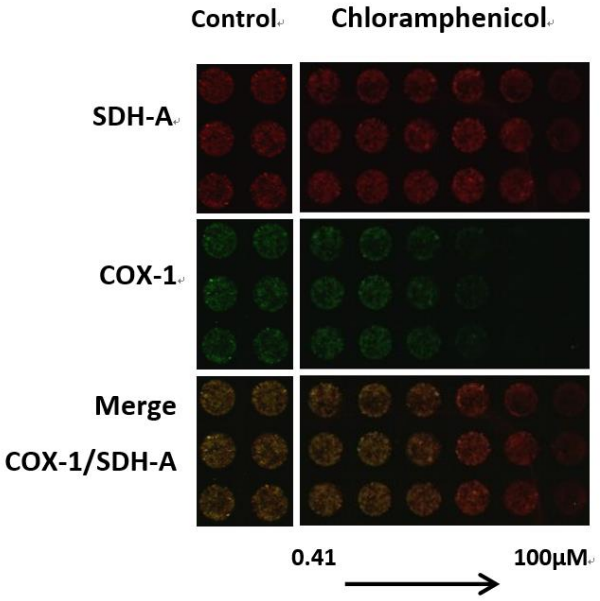
Compound(s)	Electron Transport Chain (ETC) Target	Effect on OCR
Oligomycin	ATP synthase (complex V)	Decrease
FCCP	Inner mitochondrial membrane	Increase
Rotenone/antimycin	Complex I and III (respectively)	Decrease



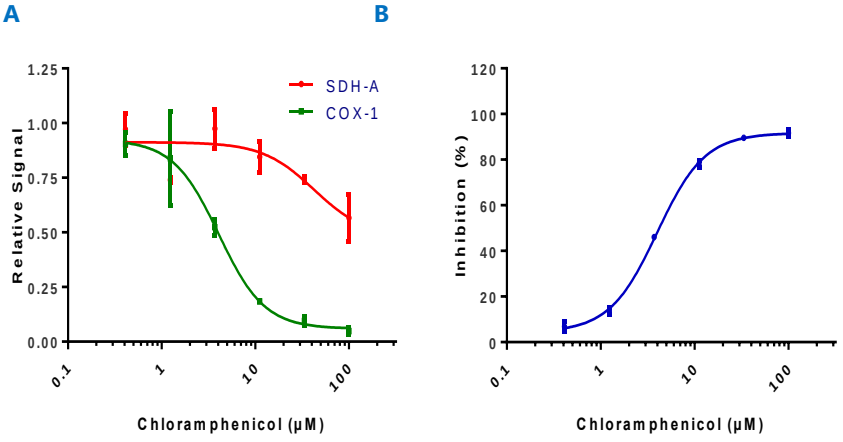
MitoBiogenesis Assay

Determination of the mitochondrial biogenesis level relative to the cellular protein synthesis provides important information on potential mitochondrial toxicity. This is particularly important for antiviral and antibiotic new drug development because the similarity between mitochondrial biogenesis and bacterial/viral replication. Many such drugs can cause serious mitochondrial toxicity.

Our mitobiogenesis assay uses Odyssey (LI-COR) with MitoBiogenesis™ In-Cell ELISA Kit (IR) (Abcam). The assay has been validated on HepG2 cells and are available in both 96- and 384-well format.



The assay simultaneously measures the levels of two mitochondrial proteins, Mitochondrial DNA encoded COXI and nuclear DNA encoded SDH-A. The specific inhibition of Mitochondrial DNA encoded protein synthesis by chloramphenicol is thus easily observed.



Inhibition of mitochondrial biogenesis by chloramphenicol. (A) HepG2 were seeded at 1200 cells/well in 384 well plate, Chloramphenicol inhibits COX-I protein synthesis relative to SDH-A protein synthesis. (B) The overall mitochondrial biogenesis inhibition was calculated from the ratio of measured COX-I/SDH-A protein levels.

Lipotoxicity

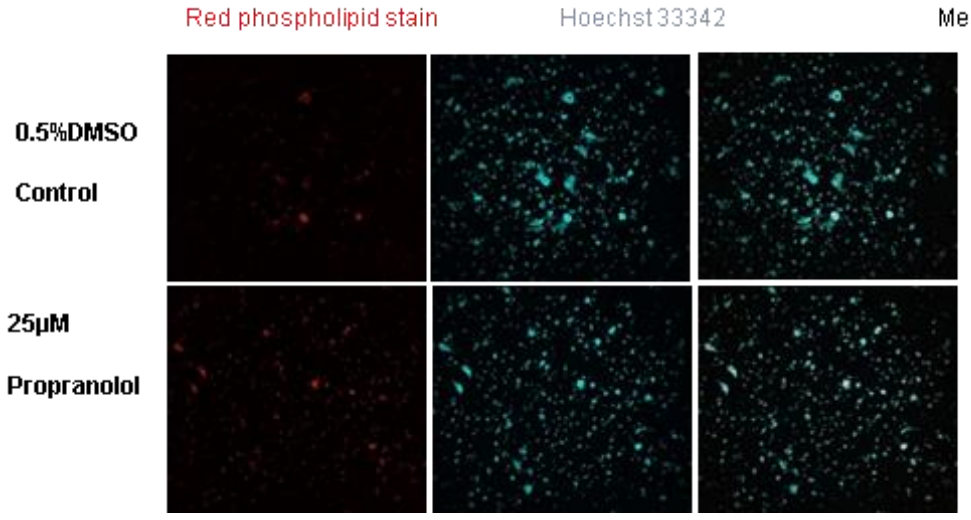
Phospholipidosis is a lysosomal storage disorder and characterized by the accumulation of excess phospholipid complexes within the internal lysosomal membranes. Cationic amphiphilic drugs (CADs), such as antibiotics, antidepressants, antihistamines and other prescription drugs, have been identified as inducers of phospholipidosis. The US FDA has acknowledged that drug-induced phospholipidosis is an adverse drug reaction[8].

Steatosis is the situation of cytoplasmic accumulation of neutral lipids. Some drug can interfere with hepatic lipid processing, leading to accumulation of triglycerides within the liver cells. This condition may lead to harmful liver inflammation, or steatohepatitis.

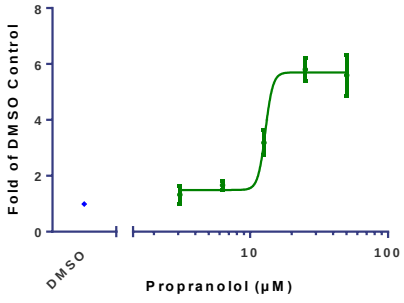
Both drug induced phospholipidosis and steatosis are often reversible conditions without remarkable consequences; however, after prolonged exposure to a particular drug, they can lead to long-term toxic effects. Therefore drug induced cellular lipotoxicity leading to phospholipidosis and/or steatosis should be evaluated during the early drug discovery stage to minimize potential risk.

WuXi AppTec now offer *in vitro* HCS assays on HepG2 cells using the HCS LipidTOX™ Stains (Thermo Fisher Scientific), with the CQ1 (confocal quantitative image cytometer, Yokogawa Electric Corporation), or Acumen Cellista (TTP Labtech) as the image reader. Phospholipidosis is detected with the LipidTOX™ Red phospholipid stain, while steatosis is detected with the LipidTOX™ Green neutral lipid stain.

Phospholipidosis assay

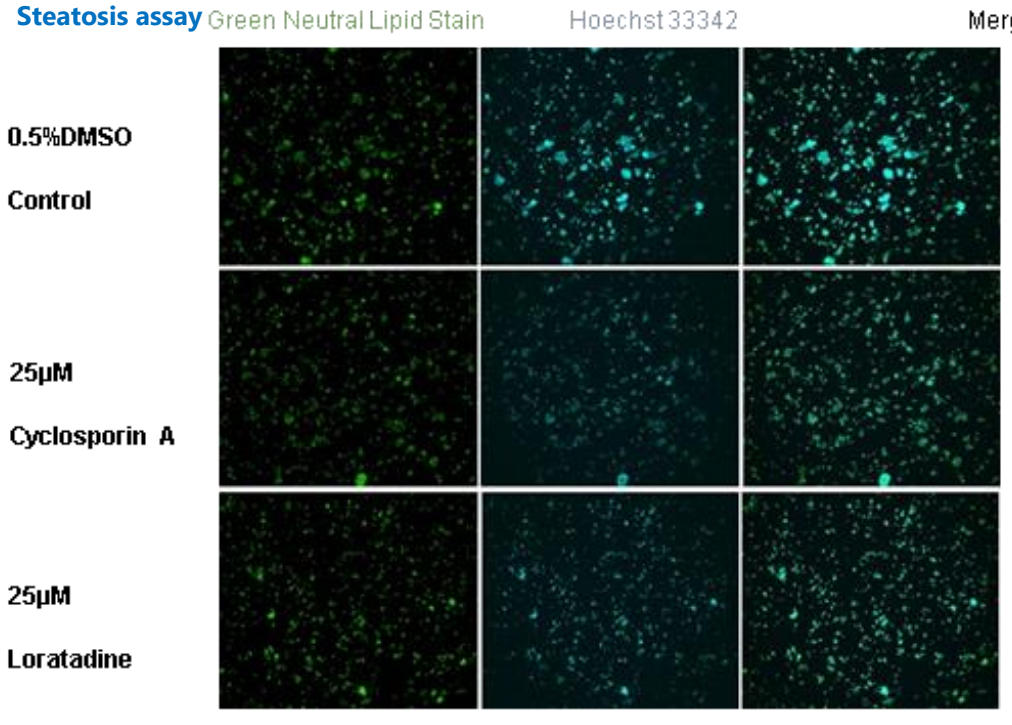


HepG2 cells were exposed to test articles and LipidTox reagent in a 96-well plate for 48 hours. Hoechst 33342 nuclear staining was used as control. Fluorescence images were captured with CQ1. LipdTox red phospholipidosis and Hoechst 33342 emit red and blue fluorescence in the same field, respectively.

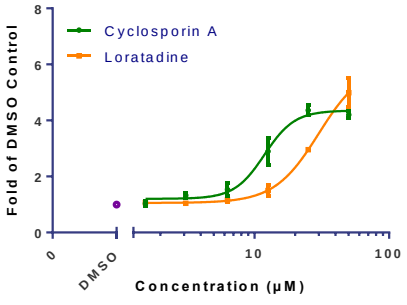


Results for red fluorescence were normalized to those of blue fluorescence (Hoechst). Propranolol concentration-dependently increased phospholipid staining.

Steatosis assay



HepG2 cells were exposed to the test articles in 96-well plate for 48 hours. Fixed cells were stained with LipidTOX™ Green neutral lipid stain and the Hoechst 33342. Fluorescence images were captured with CQ1. The Green Neutral Lipid Stain and Hoechst 33342 emit green and blue fluorescence in the same field, respectively.



Results for green fluorescence (LipidTox Green Neutral Lipid Stain) were normalized to those of blue fluorescence (Hoechst). Both Cyclosporin A and Loratadine concentration-dependently increased the Green Neutral Lipid Stain, suggesting the accumulation of steatosis.

Hepatotoxicity

The liver plays a central role in transforming and clearing chemicals and is susceptible to the toxicity from these agents. Drug-induced liver injury (DILI) is caused by many hundreds of widely prescribed drugs and is a leading cause of drug development and registration failure, withdrawal of approved drug, and cautionary labeling which restricts drug usage. The assessment of compound-induced hepatotoxicity has traditionally relied on *in vivo* studies; however these studies are limited to a small amount of late stage compounds, and the predictive value is not high enough. A recent survey demonstrated that animal models exhibit low positive predictive values of human liver toxicity with only 33% (rats), 27% (dogs), and 50% (monkeys)[9]. Since most severe DILI is due to hepatocellular injury, *in vitro* hepatotoxicity testing would provide valuable information to predict potential risk.

WuXi Biology offers multiplexed *in vitro* assays to assess potential hepatotoxicity at cellular level. The assays use HepG2 (human liver hepatocellular carcinoma) cells, or human primary hepatocytes. By using these cells as *in vitro* models, all assays listed in the General Cytotoxicity, Mitochondrial Toxicity, and Lipotoxicity sections in this brochure can be used to evaluate potential hepatotoxicity. The combination of these assays will provide valuable information from different endpoint to analyze the mechanism and severity of hepatotoxicity.

2D culture hepatotoxicity testing using HepG2 cells or human primary hepatotoxicity

Recommended HepG2 hepatotoxicity first tier assays:

- Cell Viability Assay: a very sensitive marker to detect general toxicity;
- Mitochondrial Membrane Potential Assay: an indicator of poor respiratory capacity and cell health;
- Mitochondrial Reactive Oxygen Species (ROS) assay: ROS increase may result in significant cell structure damage and cause oxidative stress;
- Caspase assay: measuring Caspase-3/7 activities.

Assay Features and Advantages:

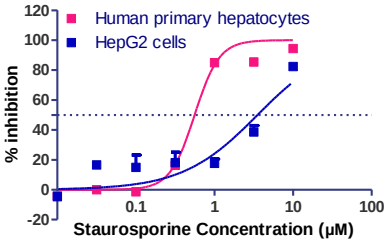
- Plate-based assay (96- or 384-well plate) read by plate reader or high content image reader;
- Medium to high throughput with high sensitivity;
- Low cost with a short turnaround time;
- Short term (24 h) and long term (10~14 d) toxicity assays available.

IC_{50} values of several reference compounds in the HepG2 cell viability assay.

	Cell Viability Assay (HepG2 cells)				
Compound	Nefazodone	Rotenone	Staurosporine	Staurosporine	Tamoxifen
Treatment	24h	24h	24h	72h	72h
IC_{50} (μM)	48.09±7.11	4.49±1.05	5.96 ± 2.32	0.036 ± 0.007	10.55±0.59
n	n=5	n=5	n=5	n=5	n=5

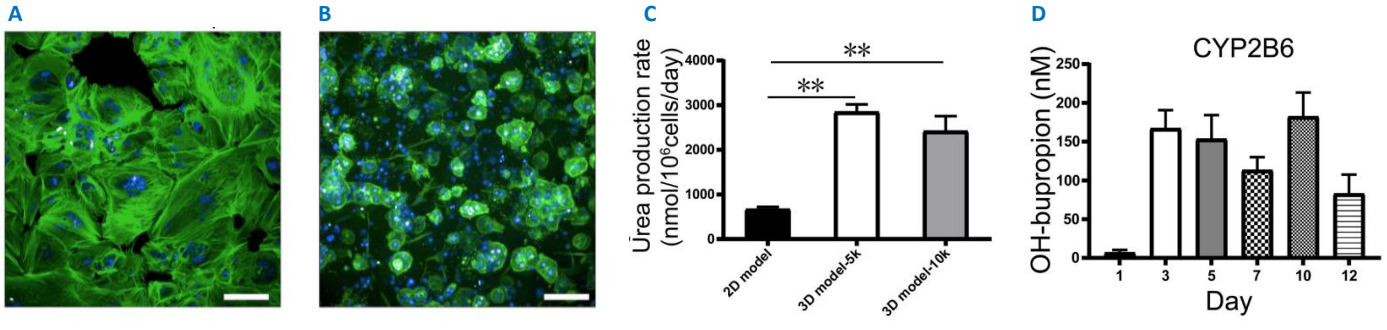
The effects of chlorpromazine and staurosporine on the viability of Human Primary Hepatocytes.

	Cell Viability Assay	
	Human Primary Hepatocytes	
Compound	Chlorpromazine	Staurosporine
Treatment	24h	24h
IC_{50} (μM)	32.53	0.56



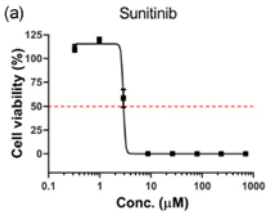
3D culture hepatotoxicity testing using human primary hepatotoxicity

A *in vitro* 3D human primary liver cellular model has been developed using human primary hepatocytes, and an integrated biomimetic array chip (iBAC, Beijing Daxiang Biotech, China) for organoid culturing. This 3D model has the advantages of high physiological fidelity and high throughput, and can accurately and effectively evaluate the potential hepatotoxicity risk of testing compounds.

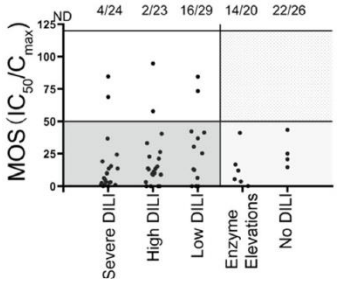


The monolayer human primary hepatocytes in 2D model were well-spread and flatten (A), whereas the 3D model formed tiny spheroids (B). The 3D model showed superior liver function as indicated by urea production compared to 2D (C). CYP450 enzymatic activities were maintained for long-term culturing on the 3D model (D). The data indicated that the 3D PHHs model can better reproduce the physiological functions of liver cells *in vitro*.

In a study on 122 FDA-approved clinical drugs with a known clinically toxicity classification, the *in vitro* 3D human primary liver cellular model showed a high positive predictive value (up to 75.7%)[10].



The concentration response curve of a representative drug (sunitinib).



Margin of safety (MOS, ratio of the IC50 and Cmax)

DILI drugs	Non-DILI drugs	TP	TN	FP	FN	Threshold	Sensitivity	Specificity	Predictivity
76	46	24	44	2	52	10x	31.6%	95.6%	63.6%
76	46	39	40	6	37	25x	51.3%	87.0%	69.1%
76	46	48	37	9	28	50x	63.2%	80.4%	71.8%
76	46	54	37	9	22	100x	71.1%	80.4%	75.7%

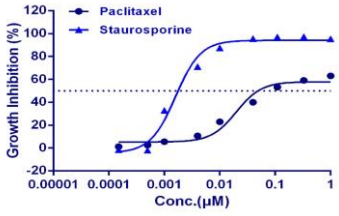
Abbreviations: FN, false negative; FP, false positive; iBAC, integrated biomimetic array chip; PHH, primary human hepatocyte; TN, true negative; TP, true positive.

Performance of the 3D model for predicting hepatotoxicity

Nephrotoxicity

The kidney's primary function is the filtration and excretion of soluble waste while retaining key biochemicals. Thus its design as a selective filter makes it particularly susceptible to toxic injury. Renal tubular cells, in particular proximal tubule cells, are especially vulnerable to drug toxicity due to their prominent role in the filtration process which exposes them to high levels of circulating toxins. Thus, our assays use human-derived cellular systems originating from the renal proximal tubule epithelial cell line (HK-2) to maximize the predictive power of nephrotoxicity. The HK-2 cells are derived from normal adult human kidneys and immortalized by transduction of the E6/E7 genes via HPV- 6.

WuXi AppTec has developed several nephrotoxicity assays to enable high throughput screening providing an early assessment of nephrotoxic effects and the potential for kidney-specific cellular injury. In general, all assays listed in the General Cytotoxicity and Mitochondrial Toxicity sections above can be used to assess nephrotoxicity, including the Cell Viability Assay, Apoptosis assay, etc.



Validation data on HK-2 cells using the CellTiter-Glo viability assay. The assay was run at 9 concentrations in triplicate and the dose response curve of paclitaxel is presented. Two reference compounds, paclitaxel and staurosporine were tested. The IC_{50} were 20 nM and 16 nM, respectively.

Hemototoxicity

The bone marrow and blood cells are common targets of toxicity from drugs. Hematotoxicity can result from direct cytotoxicity to the various blood cell types or from targeting of sensitive blood-forming stem cells/progenitor cells in the bone marrow. *In vitro* models for investigating the hematotoxicity potential of new test compounds are critical in early preclinical drug development. Hematopoietic stem/progenitor cells express the cluster of differentiation marker CD34.

WuXi Biology has developed a cell based assay to Evaluate Hematotoxicity Using Bone-marrow-derived Stem/progenitor cells Differentiating into Hekatopoietic Lineages, as described by Mahalingaiah et al (2018)[11]. The assay uses the CD34+ human bone marrow cells (ATCC). 96-well CTG-3D assay (Promega) is used in the assay, with Envision as the reader.

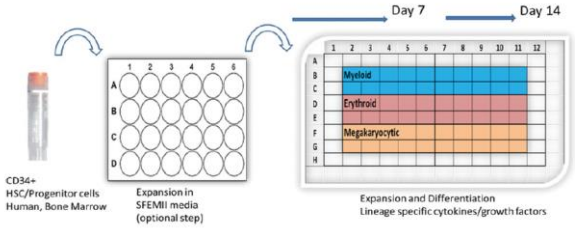
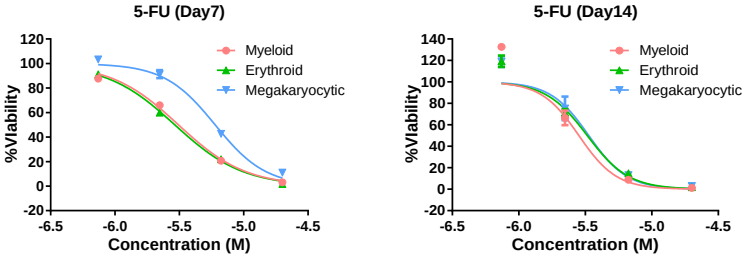


Plate map/design for seeding CD34+ cells for growth and differentiation into myeloid, erythroid, and megakaryocytic lineages.



IC50 (μM)	Megakaryocyte	Myeloid	Erythroid
Day 7	6.00	3.11	2.92
Day 14	2.83	3.36	3.27

The IC50 obtained is consistent to the literature[11].

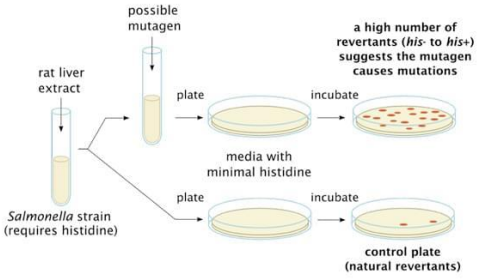
Genotoxicity

When DNA is exposed to particular chemicals, mutations and other damage can occur leading to cancer and/or teratogenic effects. The severity of these effects then necessitates examining whether new or existing chemicals intended for human use have any effect on DNA. This genotoxic potential is an integral part of the basic toxicological information package used in the decision-making and risk assessment process of drug development. Since no single test is capable of detecting all relevant genotoxic endpoints, a battery of tests for genotoxicity is recommended by regulatory agencies.

● Bacteria Reverse Mutation Assay (Ames Assay)

The Ames assay uses amino-acid requiring strains of Salmonella typhimurium and Escherichia coli to detect point mutations, which involve substitution, addition or deletion of one or a few DNA base pairs. The principle of the assay is that it detects mutations which revert mutations present in the test strains and restore the functional capability of the bacteria to synthesize an essential amino acid. The revertant bacteria are detected by their ability to grow in the absence of the amino acid required by the parent test strain.

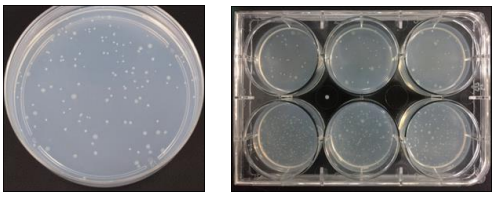
WuXi Biology offers the Ames Assay following the OECD (471) guidance[12]. The five strains of bacteria are used in the assay: TA98, TA100, TA1535 and TA1537, and *E. coli WP2 uvrA (pKM101)*.



General Procedure of the Ames Assay

Mini Ames Assay

The mini Ames assay is modified from the standard Ames test that uses 6-well plates and 20% of the typical Ames assay medium. The purpose is to generate a rapid screening test that utilizes small quantities of the test compound but still gives results in agreement with those of the standard Ames assay. The mini Ames assay is widely used as an early compound screen during lead optimization or preclinical candidate selection.



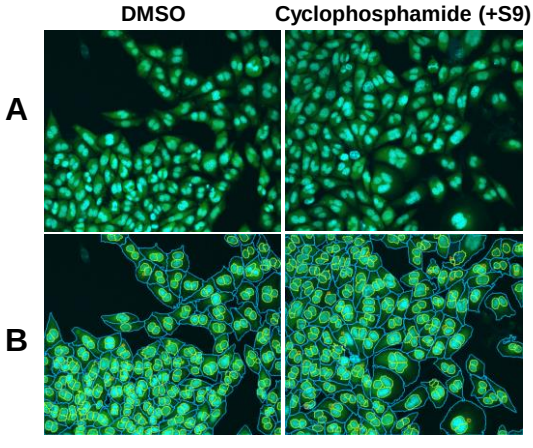
A Petri dish used in a standard Ames (left) and the 6-well plate used in the mini Ames (right).

	TA98		TA100	
	+S9	-S9	+S9	-S9
DMSO	6	13	36	26
2-Aminoanthracene (2 μg/well)	172		268	
2-Nitroflurene (4 μg/well)		224		
MNNG (1 μg/well)				671
Fold Response	28.7	17.2	7.4	25.8

Relative mutagenic potential of reference compounds as detected by Salmonella typhimurium TA98 and TA100. The numbers in the table are means in three wells.

● HCS *In Vitro* Micronucleus Assay

Micronucleus formation is a hallmark of genetic toxicity, and micronucleus assay is a test used in toxicological screening for potential genotoxic effects. The *in vitro* micronucleus assay is one of the recommended regulatory testing battery for genotoxicity. This assay detects clastogenic and aneugenic compounds, i.e. compounds which cause chromosome breakages or loss of a complete chromosome, by visualization and quantification of micronuclei in the cytoplasm. WuXi Biology provides a plate based HCS *in vitro* micronucleus assay according to the OECD (487) guidance[13]. The assay uses an automated fluorescence microscopy platform CQ1, combining with an automated pattern recognition and analysis software to detect micronucleus formation in CHO-K1 cells.

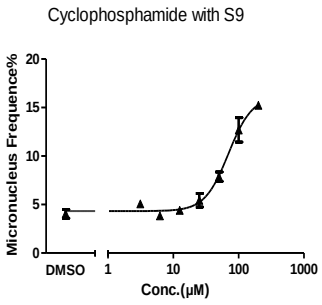


Representative images showing the effects of Cyclophosphamide (+S9) on the micronucleus formation in CHO-K1 cells.

A: original fluorescence images captured with CQ1. The cells were stained with calcein-AM (green) and Hoechst 33342 (blue).

B: the analyzed image of the same field in the CQ1 analysis software.

C



C: Cyclophosphamide (+S9) concentration - dependently increased micronucleus frequency.

D

Compounds	Data Source	Maximum % of micronucleated cells	Maximum % of cytotoxicity
Bleomycin sulfate	Literature	30	51.2
	WuXi AppTec	31.0	64.3
Cyclophosphamide	Literature	27	46.7
	WuXi AppTec	16.9	54.2

D: The comparison of WuXi AppTec data and literature data[14].

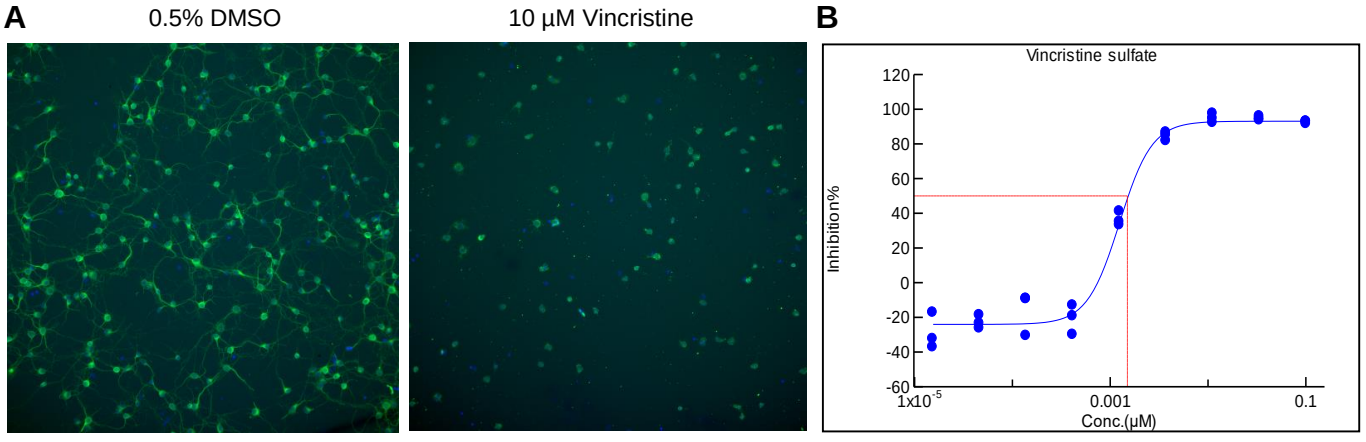
Neurotoxicity

The central nervous system (CNS) is often considered the most frequent target organ of systemic toxicity. Because of the imperceptibility of neurotoxicity, most of the project closures due to neurotoxic issues occur in the clinical phase. *In vitro* models could be useful for rapid toxicological screening of chemicals for their potential to produce neurotoxicity. Cell cultures derived from nervous system tissue have proven to be powerful tools for elucidating cellular and molecular mechanisms of nervous system development and function, and have been used to understand the mechanism of action of neurotoxic chemicals.

● Neurite Outgrowth Assay

Neurite outgrowth is the projection and extension of axonal processes from developing neuronal cell bodies that occurs via cytoskeletal reorganization, and is an important process during normal neuronal development. Through this process neurons organize themselves into highly-complex functional networks connected synaptically to one another. The complex nature of the regulation of neurite outgrowth provides a wide range of potential targets for chemical perturbation of this process. When the sites of action are unknown, the neurite outgrowth assay is suitable for hazard identification and screening for chemical-induced changes.

WuXi Biology has developed a high content analysis assay using CellInsight CX7 HCS Platform in several different cellular models, ie, NGF-stimulated PC12 cells, primarily cultured fetal rat neurons, iPSC-derived neurons. This cell-based screening assay is a good tool for assessing potential neurotoxicity.



A: Representative images showing the effects of vincristine on the neurite outgrowth from the primarily cultured rat hippocampal neurons. The cells were stained with beta III tubulin (green) and Hoechst 33342 (blue). The images were taken with CX7.

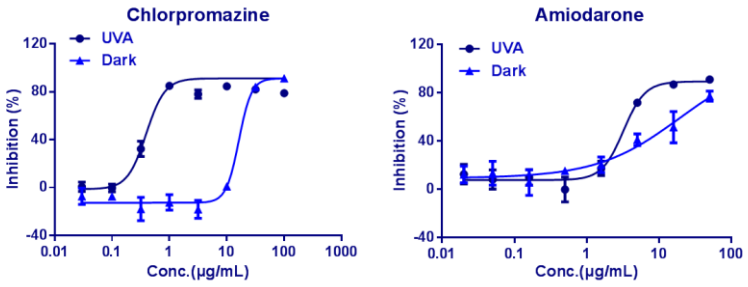
B: Vincristine concentration-dependently reduced the neurite length.

Phototoxicity

Phototoxicity is defined as a toxic response from a substance applied to the body, which is either elicited or increased after subsequent exposure to light, or that is induced by skin irradiation after systemic administration of a substance. The regulatory guidelines for phototoxicity are covered by the ICH S10[15], and the OECD Guideline for the Testing of Chemicals 432: *In Vitro* 3T3 NRU Phototoxicity Test[16].

● *In Vitro* 3T3 NRU Phototoxicity Test

The *in vitro* 3T3 NRU phototoxicity test is used to identify the phototoxic potential of a test substance induced by the excited chemical after exposure to light. The test evaluates photo-cytotoxicity by the relative reduction in viability of cells exposed to the chemical in the presence versus absence of light. Now the test has been fully validated in WuXi Biology Group using the permanent mouse fibroblast cell line, Balb/c 3T3, following the protocol documented in the OECD (432) guideline. SOL 500 (Dr. Hönle AG) was used as the light source while SpectraMax2 (Molecular Devices) plate reader was used to collect data.



Test article	IC_{50} Dark	IC_{50} UVA	PIF	Phototoxicity
	(μg/mL)	(μg/mL)		PIF
Chlorpromazine	13.21± 4.29	0.35±0.07	38.2	+
Amiodarone hydrochloride	17.30±2.61	2.79±0.59	6.2	+

Results of two phototoxic compounds, chlopromazine and amiodarone hydrochloride. For the result evaluation, the Photo-Irritation-Factor (PIF) was calculated using the following formula: PIF = IC_{50}(-UV)/IC_{50}(+UV). In this study the PIF values of the two compounds are consistent with the reference data in OECD (432) guideline. A test material is defined as having a potential phototoxic hazard if the PIF value is or greater than 5.

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