

ACEA BioSciences, Inc	ACEA Biosciences, Inc. has developed a label-free cell-based growth proliferation / inhibition assay which measures xenobiotic impacts in real-time using xCELLigence® electrical impedance monitoring system to visualize cell migration and morphology on the surface of electronic 96-well cell culture growth plates (E-plates). This platform allows for monitoring dynamic cellular responses to chemical exposures in real time, recorded as changes in cell adhesion and morphology at the electrode: solution interface measured automatically using electronic microsensors. Changes in electrical impedance can be monitored over time to help visualize dynamic responses as well as acute toxicity and cytotoxic effects, making this test platform an efficient high-throughput technology for in vitro investigation of potential xenobiotic bioactivity.
Apredica (Evotec)	Apredica assays used the CellCiphr® High Content Screening to investigate the potential for xenobiotic impacts on multiple key cellular endpoints including mitochondrial function, cytoskeletal pathways, cell cycle progression, nuclear changes, DNA damage, stress pathways, and cell viability. The cellular systems biology (CSB) approach is used to screen human liver cells (primary hepatocytes and HepG2) containing elevated xenobiotic biotransformation potential compared to other in vitro models. This high-content imaging platform uses a panel of fluorescence-based assays to quantify multiple functional biomarkers of hepatotoxicity over time and provides an efficient assessment of endpoints important for hepatic cellular function in 384-well plates.
ARUNA	The ToxCast assays conducted in cooperation with ArunaA Biomedical partners are designed to investigate chemical potential for developmental neurotoxicity (DNT) in human embryonic stem cell lines. H9-derived cells are locked at different neuronal developmental states of interest to DNT investigations of chemical exposures. These assays monitor human embryonic neuroprogenitor (hNP) and neural crest (hNC) cells for growth and survival using a migration assay which tracks the presence/absence of viable nuclei movement into a defined circular area in each microplate well, and neural net cells (NN) are assessed for chemical responses in neurite outgrowth assays which monitor changes in neurite length and number of branch points (both total number of branch points and number formed per neuron). These different measurements are assessed following 72 hour incubations with test chemical to help predict the potential to disrupt neurogenesis in developing human embryos.
Attagene, Inc	The Attagene assays track changes in transcription factor (TF) activity in response to chemical perturbations by utilizing a library of multiple reporter transcription unit (MRTU) constructs regulated by individual transcription factor response elements. This family of Attagene assays employ a recently developed profiling technology (FACTORIAL) which consists of both cis-regulating element (promoter) binding by RTUs (CIS format), and trans-acting TF DNA binding sites (TRANS format). The MRTUs are transfected into an in-house clone of human liver hepatoma cell line HepG2 (variant HG19), and while the CIS- format assay measures changes in RTU expression resulting from TF binding to response element DNA-binding sites, for the TRANS assays each RTU expresses a chimeric GAL4-NR protein that regulates transcription of a reporter sequence. Response to 24-hour incubation of test chemicals with cells in a 24-well plate is monitored by isolating mRNA followed by cDNA synthesis, fluorescent labeling, PCR amplification, restriction enzyme digestion and quantitation of individual RTUs with capillary electrophoresis.

BioReliance	<p>The BioReliance In Vitro Micronucleus assay is a high-throughput cell-based genotoxicity assay conducted in 96-well plates using Chinese Hamster Ovary (CHO) cell line to examine chromosomal damage induced by chemical exposures. This assay screens compounds for genotoxic effects by detecting small, extra nuclei in the cytoplasm which represent chromosomes or chromosome fragments excluded from the nuclei during cell division. Multiple cellular parameters, including cell cycle, relative survival, and membrane integrity are measured simultaneously with micronuclei frequency using fluorescent staining and highly sensitive flow cytometric detection. This platform allows researchers to assess system responses to chemical exposures following 24 hour exposures by visualizing chromosomal aberrations resulting from multiple genotoxic modes of action, including clastogen (DNA double strand breakage / fragmentation) and aneugen (spindle fiber dysfunction / chromosomal loss) occurrence. Standard genotoxic (mitomycin C) and pro-genotoxic (cyclophosphamide) positive control compounds provide assay acceptability data. The assay is conducted both with and without exogenous metabolic activation using Aroclor 1254-induced rat liver S9 fraction as a metabolic activation system.</p>
BioSeek (DiscoverX)	<p>The BioSeek assays performed under the ToxCast program utilize the Biologically Multiplexed Activity Profiling (BioMAP®) platform, which integrates chemical bioactivity response data from multiple complex cell culture and co-culture systems to profile hundreds of clinically relevant biomarker endpoints. The BioMAP® platform uses primary human cells from diverse sources to generate organ-specific predictions of toxicity. The cells are incubated with the ToxCast chemical library and introduced stimulants (cytokines or growth factors, for example) and following 24 or 72-h incubation with test chemical in a 96-well plate, a variety of well-characterized disease and therapeutic-based biomarkers (including cell surface receptors, cell adhesion proteins and chemokines) are measured via ELISA to detect xenobiotic-induced changes in expression levels. By leveraging cellular regulatory networks to detect chemical perturbation of common biological pathways, this platform provides a high-throughput screening approach to testing of human-relevant chemical bioactivity in diverse primary cell lines.</p>
CEETOX	<p>The CeeTox H295R steroidogenesis assays evaluate a human-derived, steroidogenically-competent adenocarcinoma cell line (H295R) in high-throughput (96-well) format to detect substances with the potential to disrupt steroid hormone production. To detect interference with steroidogenesis, this assay quantified 13 different steroid hormones using high-throughput HPLC-MS/MS. Following chemical exposure, medium was removed for steroid hormone analysis, and cells were subjected to the MTT assay as an indicator of cell viability. This assay involved a three-tiered screening strategy. The initial stage was designed to identify a maximum tolerated concentration (MTC) of test chemical in H295R cells. Following MTC determination, each chemical was screened for effects on steroidogenesis by quantifying changes in hormone levels at the MTC (single test concentration). Chemicals selected for follow-up were evaluated in dose-response to establish whether changes in hormone levels were concentration-dependent. In an initial screening exercise, chemicals were selected for concentration-response follow-up if at least 4 different hormones were significantly affected when screened at the MTC. Concentration-response experiments were conducted using six concentrations, serially diluted down from the MTC at half-log increments.</p>

CellzDirect	<p>The ToxCast CellzDirect assays used metabolically competent primary cultures of human hepatocytes to monitor xenobiotic perturbations of multiple liver-relevant pathways. This platform used quantitative Nuclease Protection Assays (qNPA) to simultaneously monitor an array of liver-relevant gene targets, primarily those regulated by nuclear receptors which serve as sentinels for key toxicant response mediated pathways; including gene products involved in absorption, metabolism, disposition and excretion of endogenous and foreign chemicals. In addition to measuring gene expression levels, visual observation of changes to cellular morphology (relative to vehicle control) are assessed at multiple time points (0, 6, 24, and 48 h) to quantify morphological alterations such as changes in cell shape, nucleus size/shape, cytoplasmic alterations, accumulation of vacuoles etc. resulting from xenobiotic exposures. mRNA induction data is transformed to log2 fold induction over DMSO (vehicle control) signal and 5-point concentration response curves were generated following 48 hours of continuous exposure to test chemicals. Performance was compared to known inducers of xenobiotic response (e.g., phenobarbital and rifampicin) and response data were generated at 4 different time points during the test duration (0, 6, 24 and 48 hours).</p>
<p>Life Technologies / Expression Analysis</p>	<p>The LTEA-assay series was developed to explore the extent to which liver specific functions and enzymes can impact toxicant bioactivity in human hepatocytes. These assays assess toxicogenomics in a metabolically competent human liver cell line (HepaRG) by screening the EPA ToxCast chemical library for gene-specific perturbations. Many xenobiotics impact biological systems following biotransformation in the liver, but most human hepatocyte cell lines express variable or negligible levels of liver-specific functions and P450 enzyme-related activities, making them unrepresentative models for in vivo toxicity. Life Technologies exposed differentiated HepaRG cells in duplicate 96-well plates to 8-point half-log dilutions of test and control chemicals, and each assay includes positive controls for nuclear receptor activation (phenobarbital). Each plate assessed cell lysis (as measured by LDH release) following 48-hour incubation with a metabolically-activated cytotoxic agent (Aflatoxin B1) and concentration-dependent responses following 48-h chemical incubation. Treated cells were lysed and frozen and each plate was immediately shipped to Expression Analysis (Quintiles) labs for qRT-PCR analysis (Fluidigm qPCR system) of changes in transcription levels for 93 genes related to biotransformation enzymes, nuclear receptors and NR mediated transporters, cell cycle regulation, and stress responses.</p>
NovaScreen	<p>The NovaScreen assays conducted as a part of the ToxCast program are cell-free binding and enzymatic activity assays which examine a diversity of biological relevant endpoints related to enzyme activity (ENZ), nuclear receptor (NR) ligand-binding, activation, distribution, metabolism and excretion (ADME) potential and G-protein-coupled receptor (GPCR) interactions, and other high value bioassay targets. High-throughput screening of in vitro chemical-target interactions across a wide variety of compounds through a broad range of biochemical interactions will help describe the bioactivity space for chemicals with limited available information. NovaScreen biochemical high-throughput screening offers preliminary evidence for chemical targets in a cell or tissues which, when combined with information from literature or targeted in vivo studies, can indicate potential pathways for toxicity.</p>

Odyssey Thera Odyssey Thera (OT) developed high-throughput chemical screening assays which utilize diverse technologies to screen protein expression and interactions in stably transfected cell-lines. OT has specialized in the development of Protein Complementation Assays (PCAs) assays which monitor key nodes in biochemical pathways. Interaction of the tagged key proteins of the node brings separate protein fragments into close proximity and reconstitutes a functional reporter (fluorescent protein) producing a fluorescent signal when the pathway is unimpeded. Changes in protein complex interactions can be impacted by a variety of biochemical events within a pathway, and these assays are designed to track xenobiotic induced changes at the level of cell functioning which may occur at multiple points along signaling pathways following incubation with test compound in 384-well plates.

STEMINA The ToxCast Stemina assays are designed to measure potential developmental toxicity in pluripotent human embryonic cells using a metabolomics-based platform. Undifferentiated H9 stem cells are exposed to test chemicals in 96-well plates for 3-day exposures followed by LC-MS metabolite analysis of changes in ornithine and cysteine levels. Healthy, well-functioning cells secrete ornithine (ORN) into surrounding media and consume cysteine (CYS). A decrease in the ratio of ORN/CYS resulting from lower CYS utilization and decreased ORN production can be indicative of a metabolic imbalance resulting from cell stress. C¹³-labeled spike-in standards for ORN/CYS are used to assess the level of test chemical exposure that alters metabolism in H9 cells with the minimal concentration causing a significant change concentration expressed as the developmental toxicity potential (dTP). In these assays, the dTP is provided as the 'teratogen index' and is the concentration of test chemical which causes the ORN/CYS ratio in H9 embryonic stem cells to fall below 0.88.

Tox21 The Tox21 quantitative high-throughput screening (qHTS) robotic platform is capable of fully-automated (hands-free) assay execution (liquid dispensing and aspiration, plate centrifugation and incubation, et cetera) and signal recording (plate readout) using 1536-well plates as a platform for the assay. This highly efficient platform allows for each compound in the large Tox21 chemical library (approximately 10,000 compounds, including ~3000 ToxCast compounds) to be tested at multiple (usually 15) concentrations. This allows the quick production of pharmacologic concentration-response relationships – the system is capable of screening around 40 plates per day – on previously untested compounds, while producing results with the accuracy of lower throughput technologies. The assays are initially developed and validated in 96- or 384-well plates, and when performance criteria are within acceptable levels the assay is miniaturized and screened 3 times against a validation library. Assay targets probe a diversity of toxicologically relevant endpoints, including overall cellular health (apoptosis induction, DNA damage, overt cytotoxicity), perturbation of cell stress signaling pathways, inflammatory response induction, and nuclear receptor modulation. Robotic screenings of the Tox21 10k library are run three times on three separate days in the qHTS format across 15 concentrations, followed by data processing (normalization and curve fitting) and data release to project partners and publication to publically accessible repositories.

VALA Vala Sciences provide High-Content Screening (HCS) assay development and cellular imaging tools to help visualize cellular responses to ToxCast chemical

exposures. Vala designed multivariable imaging assays, reagents and protocols using combinations of fluorescent stains and custom antibodies to monitor an array of cellular biological processes and pathways. Vala assays have included targets which examine adipogenesis/lipolysis in human pre-adipocytes and assays which examine angiogenesis/tubulogenesis in human HUVEC. Using customized cell-based instrumentation and analysis (CyteSeer software) to define cellular organelle imaging and identify co-localization parameters, these assays are conducted in 384-well plates to screen multiple changes in cellular morphology; changes in organelle size, shape and distribution; and cytotoxicity across a concentration-response gradient.

ZF_NHEERL As part of the US EPA's efforts to identify chemical compounds with elevated potential to trigger adverse biological effects in exposed systems, the agency's NHEERL and NCCT labs have collaborated to screen the ToxCast chemical library for developmental effects in zebrafish (*Danio rerio*) in medium-throughput assays. Zebrafish provide a rapidly developing and easily maintained test organism which is visually transparent through much of its embryonic development and has an elevated xenobiotic biotransformation potential when compared to other commonly used models of developmental toxicity. These assays screened embryonic responses to chemical exposures in 96-well plates by visually assessing multiple phenotypic indicators of developmental interference, including malformations, failure to hatch, and mortality. Each compound was initially tested at a relatively high single concentration (80 μ M) followed by a rigorous concentration-response screening which tested a 11-point concentration range spanning 5 orders of magnitude to assess chemical potency. Embryos were exposed to test chemicals for 120 hours, and developmental defects were quantified through optical microscopy at 144 hours post fertilization (hpf). Many key developmental signaling pathways and their regulatory mechanisms are conserved between fish and mammals, making zebrafish toxicity assays a unique integrative model of embryogenesis which is highly adaptable to medium-throughput toxicity screening platforms such as these methods developed for the ToxCast program.

ZF_Tanguay These assays were conducted in collaboration with the US EPA's ToxCast program and the Tanguay Labs at Oregon State University to help identify compounds with the capacity to interfere with embryonic development. Cell culture systems are limited in their capacity to express gene products and may lack the ability to metabolize xenobiotics. The zebrafish (*Danio rerio*) has thus become a valuable model system for the testing of chemical exposures in high-throughput screening platforms, as this provides a small but complex level of biological organization amenable to testing multiple developmental processes in high-throughput platforms, using 96-well microtiter plates. Zebrafish share high (70%) genetic homology with humans, and approximately 84% of human disease-associated genes are also occur in zebrafish. To assess chemical response, fertilized zebrafish embryos were enzymatically dechorinated at 4 hours post fertilization (hpf) to enhance compound bio-accessibility and each embryo was placed one fish per well using an automated embryo placement system at 6 hpf. Embryos were statically exposed to test compounds until 120 hpf. Each compound was tested in a broad concentration range spanning more than 4 orders of magnitude with multiple (8) replicates at each concentration. The platform allows for simultaneous evaluation

of 22 biological endpoints, including morphological assessments and functional response indicators as well as measurements of direct mortality.