Call for Nominations of Quantitative High-Throughput Screening Assays from Relevant Human Toxicity Pathways

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Abstract:
The National Research Council of the United States National Academies of Science has recently released a document outlining a long-range vision and strategy for transforming toxicity testing from largely whole animal-based testing to one based on in vitro assays. "Toxicity Testing in the 21st Century: A Vision and a Strategy" advises a focus on relevant human toxicity pathway assays. Toxicity pathways are defined in the document as "Cellular response pathways that, when sufficiently perturbed, are expected to result in adverse health effects". Results of such pathway screens would serve as a filter to drive selection of more specific, targeted testing that will complement and validate the pathway assays. In response to this report, the US EPA has partnered with two NIH organizations, the National Toxicology Program and the NIH Chemical Genomics Center (NCGC), in a program named Tox21. A major goal of this collaboration is to screen chemical libraries consisting of known toxicants, chemicals of environmental and occupational exposure concern, and human pharmaceuticals in cell-based pathway assays. Currently, approximately 3000 compounds (increasing to 9000 by the end of 2009) are being validated and screened in quantitative high-throughput (qHTS) format at the NCGC producing extensive concentration-response data for a diverse set of potential toxicity pathways. The Tox21 collaboration is extremely interested in accessing additional toxicity pathway assays that can be conducted in qHTS format. Nominations for such assays are currently being sought from all interested sectors--industry, universities, interest groups and the public. Accepted nominations of the assays could be screened at NCGC with results made available to the public through the PubChem database or other databases, including ACToR and CEBS. It is hoped that the opportunity to evaluate robust, qHTS assays against these large libraries of important compounds will contribute toward a better understanding of what constitutes a toxicity pathway and ultimately result in a stronger scientific foundation for understanding risk to humans of exposure to chemicals of all types. *This work was reviewed by EPA and approved for publication but does not necessarily reflect official Agency policy.*

Collaboration Partners

qHTS Assay Guidance Criteria

Criteria	Cell-based
Plate Format*	96-well or higher density plate NCGC 1536-well format Assay volume 4-6 ul
Assay Steps	≤ 10 steps with 96-well plate. Steps include, reagent additions, timed incubations, plate transfers to incubator, reading, etc.
Minimum time increments and maximum assay duration	<24 hr is ideal; max48 hrs. Minimum assaywindow is 5 min.
Reagent removal steps*	Aspiration steps*
Temperature	Between RT and 37°C
Demonstrated DMSO Tolerance*	0.5 – 1% DMSO
Signal : Background Ratio	≥ 3 fold
CV across the DMSO plate	≤ 10%
Day-to-Day variation of control (e.g., IC_{50} EC_{50})	<3-fold
Reagent stability @ final working concentration	>8 hrs @ RT or on ice bath; No on-line thawing
Protocol	Complete detailed protocol. All steps, equipment used, all vendor & catalog # for reagents. Detailed cell culture procedure, passage #. Data from 96-well or high density plate tests.
Detectors	**PE ViewLux** (Top reading only: FI, TRF, FP, Abs, Luminescence) **PE Envision** (bottom reading FI, ALPHA) **Acumen Explorer** (fluorescent laser cytometry)
Special	Cells must be certified mycoplasma-free by direct culture assay and cell-DNA fluorochrome staining.

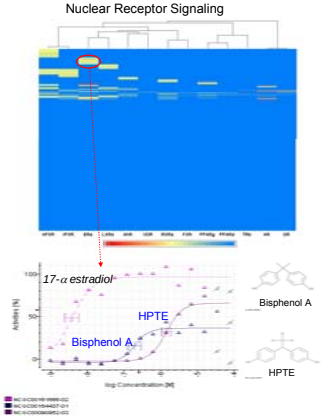
Table Notes:
* NCGC will convert assays to 1536-well from 96- or 384-well format.

* Reagent removal steps: Are any step that requires the removal of material from the well of a microtiter plate. While such steps may be routine with 96-well plates they are not recommended on robotic systems using 1536-well plates.

* Demonstrated DMSO Tolerance: Because all compounds screened are stored in ~100% DMSO and delivered as a 1:100 dilution to the assay the assay sensitivity to between 0.5 and 1% DMSO must be determined.

Example qHTS Results:

Nuclear Receptor Signaling



Assay Nomination Form

- Available from:
  - http://ntp.niehs.nih.gov/go/htsnom
  - http://epa.gov/ncct/toxcast/assays.html
- Nominations accepted from government, private, academic, NGO sectors
- Accepted nominations may be run at NCGC
- Results will be publically available via PubChem

Tox21 Assay Nomination Form:

Date:

Name:

Organization:

Contact Information:

Assay Name:

Biological/Toxicity Pathway:

Relevance to Tox21:

Critical Factors for Assay Success:

Assay Technology:

Assay Source:

Assay Format:

Reference Compounds:

Validation Status:

Major Estimated Costs:

Example:

Tox21 Assay Nomination Form:

Date: 22Aug08

Name: Keith Houck
Organization: EPA/ORD/NCCT
Contact Information: houck.keith@epa.gov 919-541-5519
Assay Name: Hedgehog signaling

Biological/Toxicity Pathway: Hedgehog Pathway. Toxicity pathway inferred; no in vivo validation as toxicity pathway.

Relevance to Tox21: The hedgehog (Hh) signaling pathway is critical to proper development of organisms ranging from flies to humans. It functions to regulate pattern development during embryogenesis and metamorphosis. Genetic studies show major effects on brain, skeleton, musculature, gastrointestinal tract and lungs in knock-out mice. Hh is a secreted protein ligand that binds to the Patched cell-surface receptor, relieving inhibition of the Smoothened receptor (SMO). SMO subsequently relays intracellular signals resulting in the activation of the transcription factor family GLI and subsequent changes in gene expression. While environmental chemicals have not been shown to affect this pathway, a plant natural product, cyclopamine, is a potent inhibitor of Hh binding to PTCH resulting in cyclopia in offspring of gestating mammals consuming the plant.

Critical Factors for Assay Success: Cell should contain appropriate signal transduction machinery. Positive response to Hh stimulation ensures at least minimal components present. Inhibition assay may be subject to false positives due to cytotoxicity. Validation data from Invitrogen done with 0.5 hr pre-incubation with compounds before adding Hh.

Assay Technology: Beta-lactamase reporter gene under control of GLI response element in NIH3T3 stable cell line.

Assay Source: Invitrogen (http://tinyurl.com/5vt6d2).

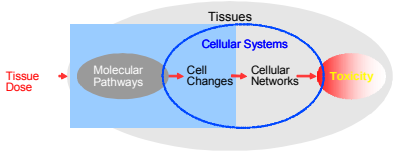
Assay Format: Should be 1536-well compatible. Validated in 384-well.

Reference Compounds: Agonists: m5ihh used to stimulate pathway (available commercially). Positive control inhibitors: Cyclopamine (EMD #239804), tomatidine (EMD #614350), SANT-1 (EMD #55303), or Purmorphamine (EMD #540220).

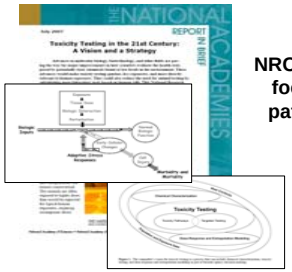
Validation Status: Invitrogen validation package. Z' of 0.67, response ratio of 4-5 in 384-well plates, DMSO tolerance fine.

Major Estimated Costs: Cell line $9900. BLAM reagents. Agonist and inhibitor purchase.

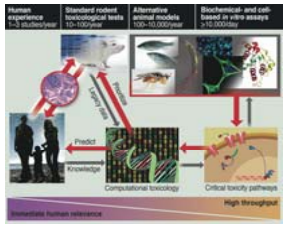
Grand Challenge in Toxicology



How do we predict human toxicity?



NRC Report: focus on pathways



Collins et al., Science 319, 906 (2008)

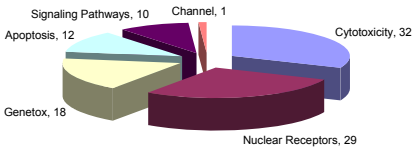
Tox21 Existing and Candidate Chemical* Screening Library

Universe	13,247
With structures	8,277
Plausible P-chem (logP)	7,116

Library	Current	Additional
NTP	1353	1400
EPA	1330	2800
NCGC		3000 (drugs)
Total	~2400	~10,000

*Sources include NTP, EPA HPV, CCL, OPPIN, OW, Inerts, ToxCast, DSSTox, EU Carcinogenomics, Pharmaceuticals, and others

Tox21 Assays Completed

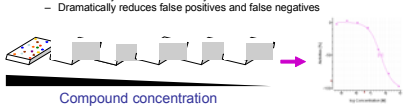


Key Questions for qHTS of Toxicity Pathways

- How to identify appropriate cellular toxicity pathways and targets
  - Target major pathway hubs?
  - Probe specific disease-related pathways?
  - How many targets needed to define a pathway?
- How to meet the need for biotransformation capacity of assay systems
  - Co-culture with primary hepatocytes?
  - Stem-cell derived hepatocytes?
  - Phase I/Phase II enzyme systems?
  - Bioreactors with metabolic capacity?

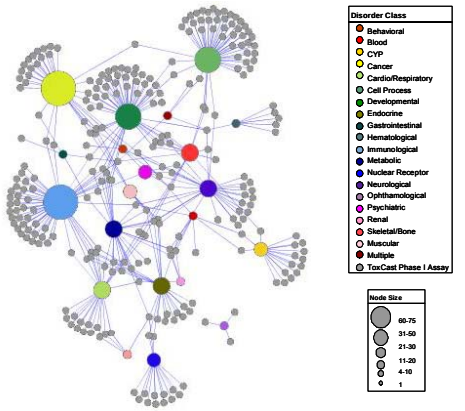
Quantitative HTS Guidlines

- Conventional HTS done at one concentration (typically 10 uM)
- qHTS assays compounds at multiple concentrations (0.5nM-92uM)
  - 7-15 concentrations
  - Assay volumes miniaturized into ~5 uL and 1536-well plate format
  - Concentration-response curve generated for each cpd from 1° screen
- Informatics pipeline for data processing, curve fitting & classification, extraction of SAR
- Generates pharmacological actives rather than statistical "hits"
  - Dramatically increases reliability
  - Dramatically reduces false positives and false negatives



Toxicity Pathways

Definition: *"Cellular response pathways that, when sufficiently perturbed in an intact animal, are expected to result in adverse health effects."* (Toxicity Testing in the 21st Century: A Vision and a Strategy (National Academies Press, Washington, DC, 2007)



Network diagram of the disorder classes probed by ToxCast Phase I HTS Assays. Colors denote grouped disease types defined by OMIM disease-gene associations and based loosely on disease classes used by Goh *et al.*, PNAS 104: 8685 (2007). Node size corresponds to the number of distinct loci present in each disorder class.

United States Environmental Protection Agency