Using automation to support validation at ECVAM

http://www.remanet.net/

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EU Reference Laboratory for Alternative Methods to Animal Testing

Outline

- Motivation.
- HTS/HCA facility at ECVAM.
- Factors to consider when automating assays.
- Case studies: 3T3/NRU and LUMI-CELL.
- Conclusions and outlook.

Motivation

Exploiting assay automation to support validation:

- Efficiency – generate data faster.
- Coverage – test broader chemical domain.
- Precision – minimise technical variance.
- Application – make ready for industrial use.
- Necessity – validation of HTS-specific assays.

HTS/HCA facility

Automation platform

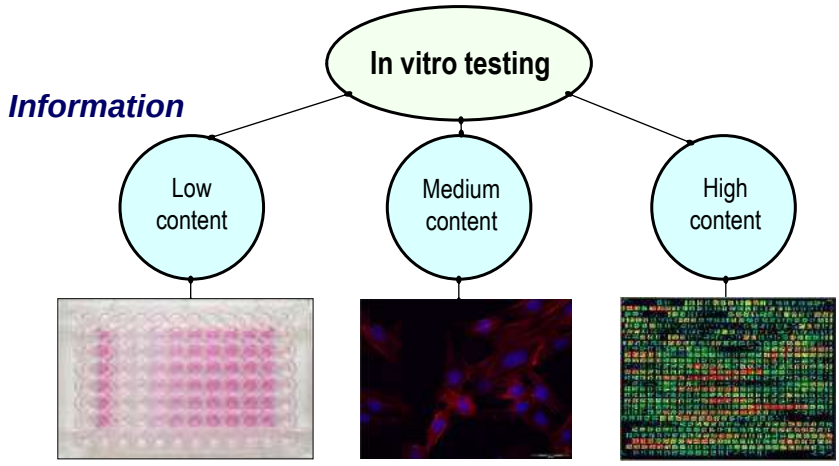
1 to 300µl volume handling

Analytical chemistry - NMR

- Verify chemical identity by checking NMR spectrum against structure.
- Determine absolute conc. in solvent.
- Determine absorbed water content.
- Asses purity and stability of chemicals.
- Detect chemical-reagent interactions.
- Routine QC-screening of libraries.
- Non-destructive thus sample is available for further analysis (MS).

Avoid spurious results, pointless retesting, and endless discussion !

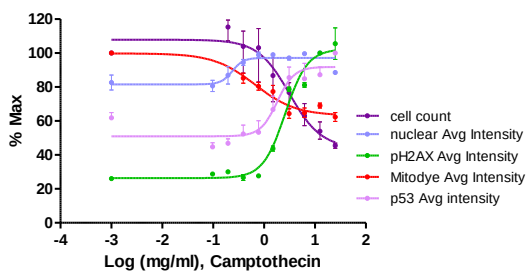
Information content



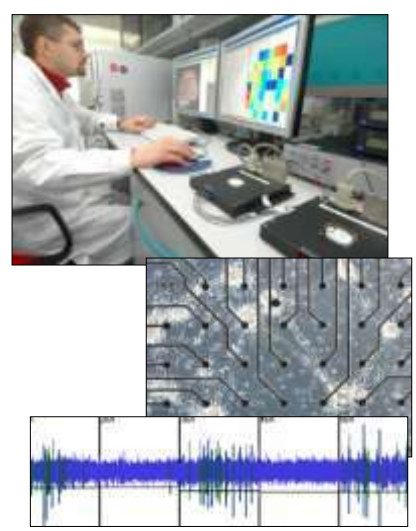
Toxicity Screening ⟶ Toxicity Profiling

High Content Analysis

Automated quantitative imaging

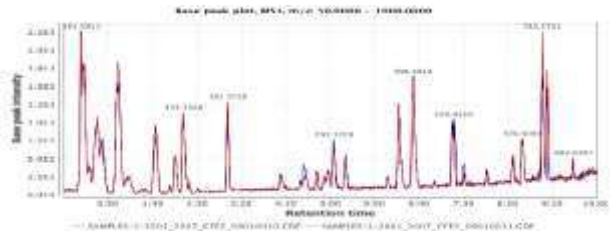


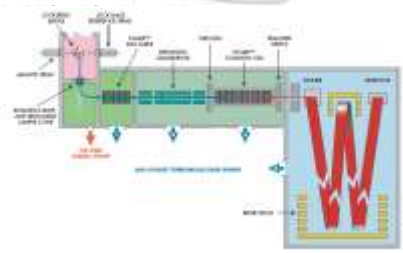
Electrophysiology using MEA

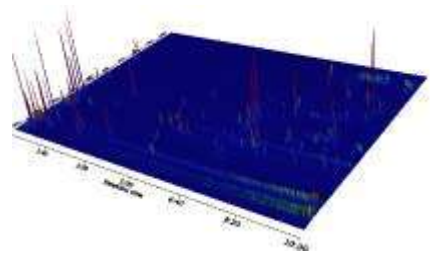


Metabonomics

Advanced MS and NMR







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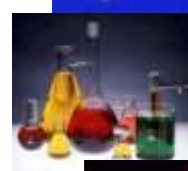
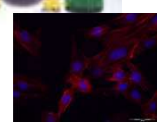
Technical aspects

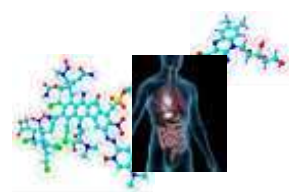
- Automation
  - liquid handling; plate manipulation, cell seeding, detection, timing
- Experimental design
  - plate format typically 96, 384, 1536 wells
  - number and position of chemicals/controls on one plate
  - conc-resp within or across plates within a run
  - degree of replication within and between runs
  - pipetting volumes and dilution protocols
- Preparation
  - chemicals prepared as DMSO stock solutions beforehand
  - volume of cells typically to fill 10 to 20 plates for a run
- Data
  - annotation, storage, retrieval, visualisation
  - normalisation, scaling model fitting





Challenges

- High setup and maintenance costs.
- High density plate formats (384 & 1536 wells).
- Handling liquids with variable phys-chem properties.
- Availability and quality of large chemical libraries.
- Scale and reproducibility of cell culturing.
- Effort and cost per experimental run.
- Operational and logistical complexity.
- Heavy price for small mistakes.



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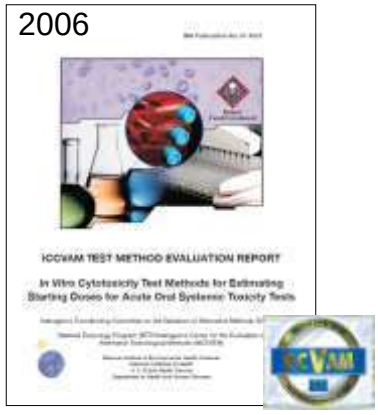
3T3/NRU Assay

2006



- 3T3 mouse fibroblasts
- 48 hour treatment duration
- EP - cellular uptake of Neutral Red dye
- 72 reference chemicals
- Stringent acceptance criteria and PS

→ Commissioning study (faithful implementation)

→ ECVAM validation study (56 chemicals)

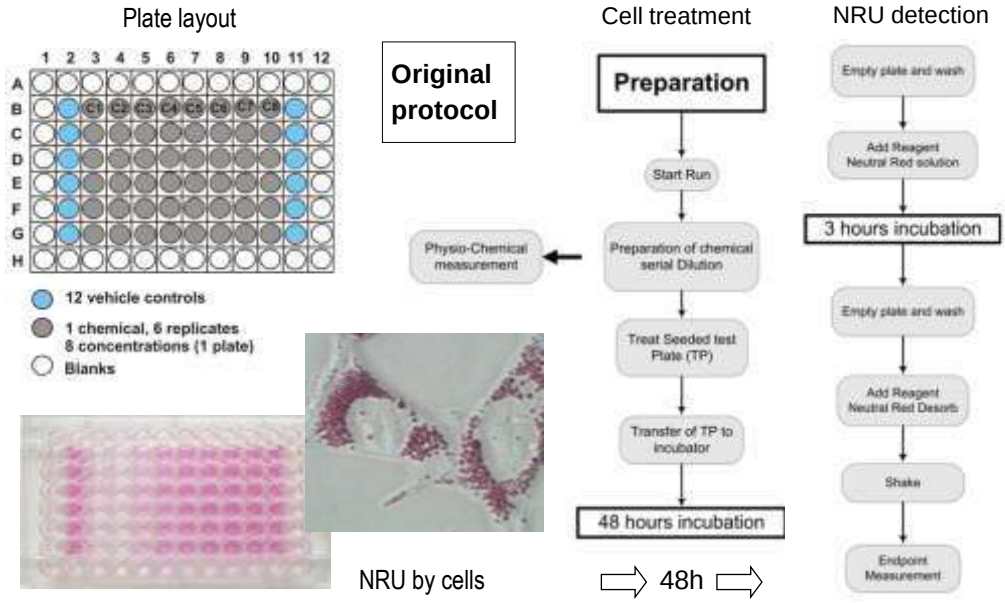
→ Scale-up (quantitative HTS format)

ICCVAM, 2006b. Test Method Evaluation Report: In vitro cytotoxicity test methods for estimating starting doses for acute oral systemic toxicity tests. NIH Publication No: 07-4519

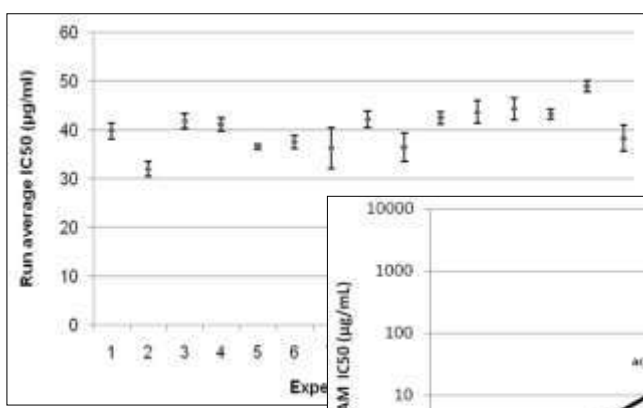
OECD, 2010. Series on Testing and Assessment, No. 129. Guidance document on using cytotoxicity tests to estimate starting doses for acute oral systemic toxicity, Paris.

Bouhifd et. al., 2011. Automation of an in vitro cytotoxicity assay used to estimated starting doses in acute oral systemic toxicity tests, submitted.

3T3/NRU protocol

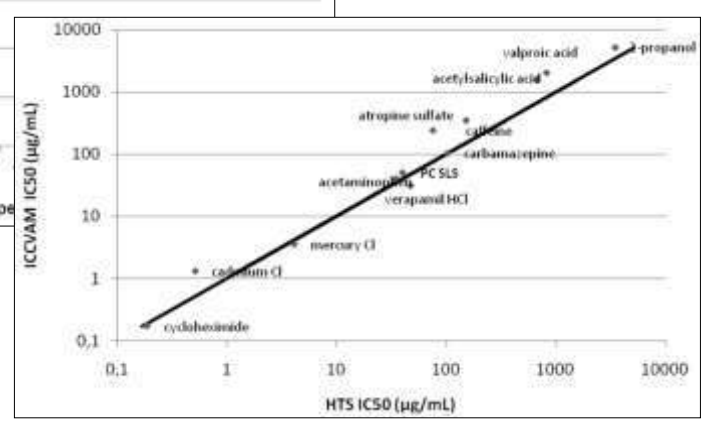


Performance Assessment



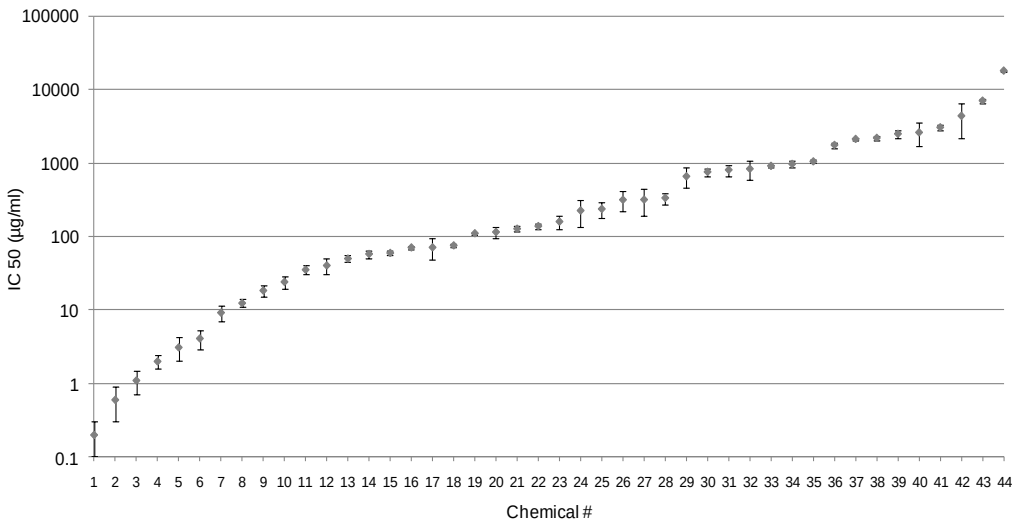
Performance standards

Historic mean IC_{50} of PC (SLS) over 6 months



Comparison of IC_{50} of 12 reference chemicals

ECVAM Validation Study



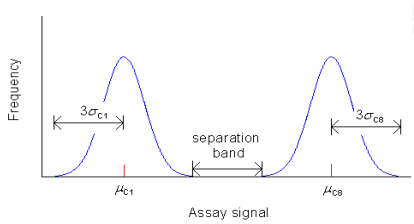
Results from 44 of 56 chemicals that could be tested and exhibited toxicity

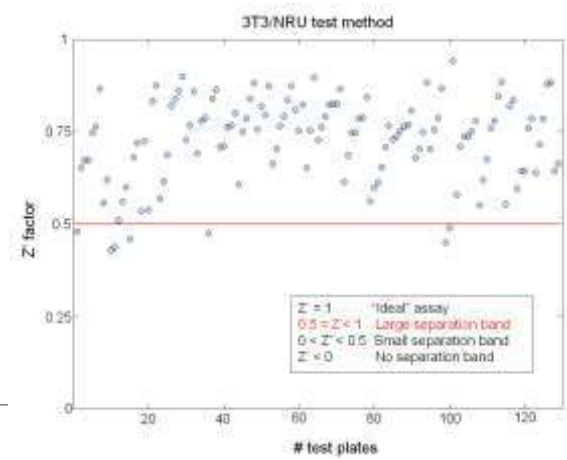
Quality assessment

Z' factor - a performance indicator for bioassays. Evaluation of assay quality

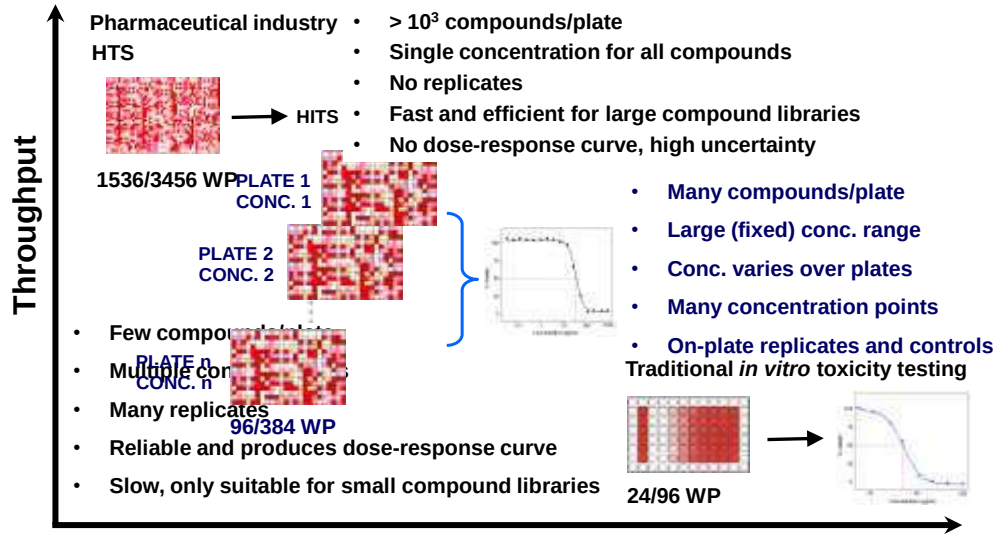
$$Z' = 1 - \frac{3\sigma_{C8} + 3\sigma_{C1}}{\mu_{C8} - \mu_{C1}}$$

C1 highest and C8 lowest conc.





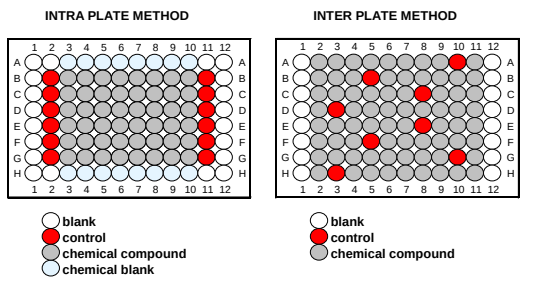
Quantitative HTS (qHTS)

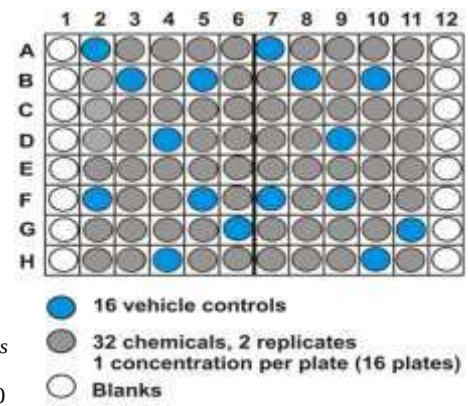


** Inglese J. et al. "Quantative high-throughput screening…" PNAS 103,11473-11478, 2006*
Xia M. et al. "Compound cytotoxicity profiling…" Environmental Health Perspectives 116, 284-291, 2008.

Quantitative HTS (qHTS)

Modelling variance in HTS experiments to estimate precision of various intra and inter plate designs (traditional versus qHTS)



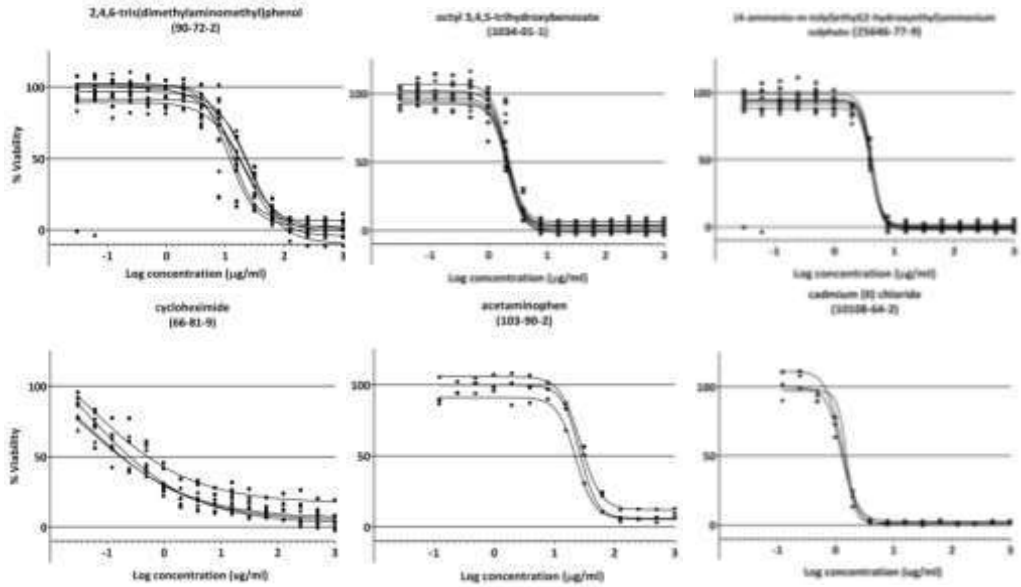


$$X_{jks} = \mu + \xi_j + \eta_{jk} + \varepsilon_{jks} \qquad Y_{ijks} = \mu + \upsilon_i + \xi_{ij} + \eta_{ijk} + \varepsilon_{ijks}$$

$$\bar{\tau}_X = Var(X_{...}) = \tau_\xi / n_J + \tau_\eta / (n_J n_K) + \tau_\varepsilon / (n_J n_K n_S) \approx 0.70$$

$$\bar{\tau}_Y = Var(Y_{...}) = \tau_\upsilon / n_I + \tau_\xi / (n_I n_J) + \tau_\eta / (n_I n_J n_K) + \tau_\varepsilon / (n_I n_J n_K n_S) \approx 0.89$$

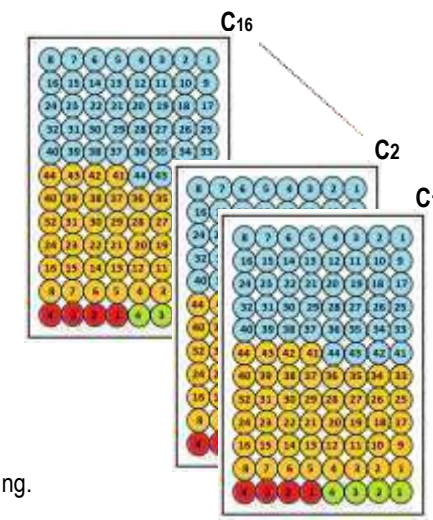
3T3/NRU using qHTS format



"LUMI-CELL" Automation

ICCVAM/NICEATM Coordinated Study

- Reporter gene assay - ER binding.
- Agonist and antagonist formats.
- 78 chemicals in test set.
- 3 test labs (manual) inc. ECVAM.
- Peer review March 2011.

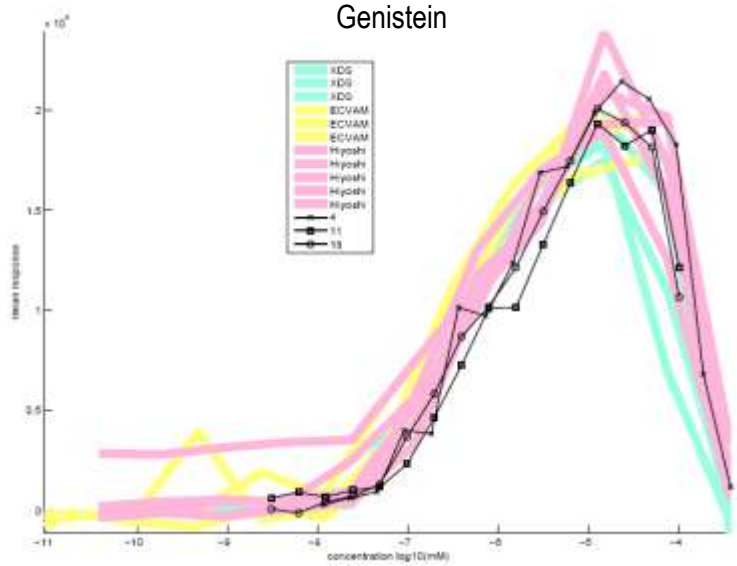


Automation at IHCP

- qHTS format with 96-well plates.
- 16 plates/concentrations, DL of 2.
- 44 chemicals per plate, 2 replicates.
- 3 biological repeats.
- pos/neg control, fixed conc, 4 replicates.
- Acceptance and normalisation criteria needed adapting.

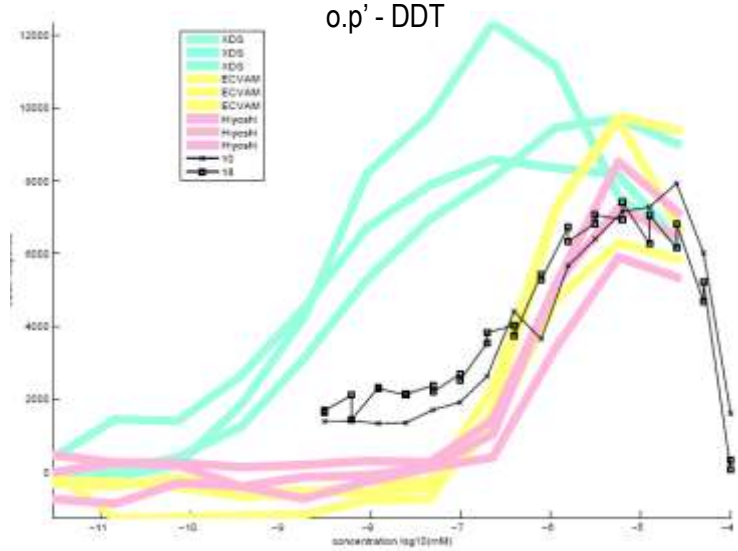
LUMI-CELL HTS Results

Genistein

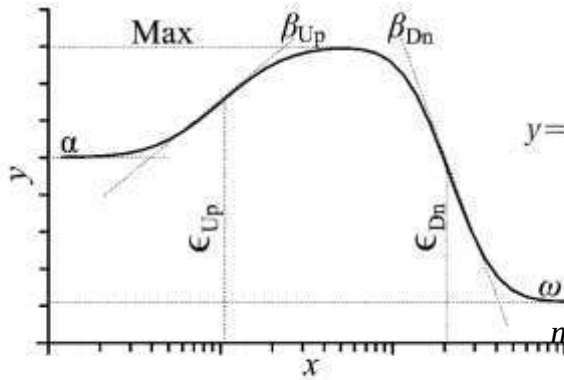


LUMI-CELL HTS Results

o.p' - DDT



Biphasic Model



$$y = \omega + \frac{\alpha - \omega + \frac{\text{Max} - \alpha}{1 + (\epsilon_{Up}/x)^{\beta_{Up}}}}{1 + (\epsilon_{Dn}/x)^{\beta_{Dn}}}$$

Modelling parameters

$max, \omega, \alpha, \beta_{up}, \beta_{dn}, \varepsilon_{up}, \varepsilon_{dn}$

FIGURE 2. Illustration of the meanings of the parameters of the general biphasic model for hill-shaped curves (eq 8).

Don't throw the baby out with the bathwater !

LUMI-CELL Automation

NIH Chemical Genomics Center

JRC000008									
JRC000015									
JRC000049									
JRC000084	Haloperidol	-			-5,885		-	-	
JRC000162	2-sec-Butylphenol	-4,759		-4,582			-5,007	-4,670	-4,778
JRC000163	4-Androstene-3,17-dione	-5,350			-6,759	-6,602	-5,143	-6,355	-6,496
JRC000164	4-cumylphenol		-7,124	-		-6,704		-6,743	-6,610
JRC000165	4-Hydroxytamoxifen	-		-		-	-	-	
JRC000166	4-Nonylphenol, mixture of isomers	-5,621		-		-5,897	-	-6,034	
JRC000167	4-tert-octylphenol	-			-	-6,983	-	-7,086	
JRC000168	6-N-Propyl-2-thiouracil	-		-	-6,156		-	-23,47	
JRC000169	a-Estradiol	-			-	-	-	-	-
JRC000170	Actinomycin D	-6,665		-		-			
JRC000172	Androstanolone	-			-	-7,093	-	-	
JRC000173	Apigenin	-5,677			-5,993	-5,606	-5,763	-5,903	
JRC000174	Apomorphine hydrochloride hemihydrate	-		-5,000			-5,332	-5,770	
JRC000175	Atrazine			-4,784					-
JRC000176	Bicalutamide	-5,611		-					-5,540
JRC000177	Bis(2-Ethylhexyl)Phthalate		2,2905	-5,029					-5,591
JRC000178	Bisphenol A	-			-6,632		-6,536	-6,710	
JRC000179	Butyl benzyl phthalate	-5,589			-5,985		-6,528	-6,019	-5,745
JRC000180	Chlordecone (Kepone)	-		-6,338	-6,643	-6,104	-	-6,117	
JRC000181	Chrysin	-5,397	-5,207		-6,136		-5,403	-5,646	-5,643
JRC000182	Clomiphene citrate saly	-		-7,048			-5,858	-6,011	
JRC000183	Corticosterone	-	-6,313		-6,535	-6,299	-6,319	-5,842	
JRC000184	Coumoesterol	-		-	-	-	-	-	
JRC000185	Cyproterone acetate	-6,048	-5,570		-5,468	-4,801	-4,630	-6,537	
JRC000186	Daidzein	-5,614	-		-5,457		-	-5,681	
JRC000187	Dexamethasone	-4,766	-4,426		-4,832		-5,693	-5,471	
JRC000188	Dibenz[a,h]anthracene	-5,316			-5,276		-4,867	-6,111	
JRC000189	Dibutyl phthalate	-5,588	-5,647		-5,774		-5,499	-5,176	
JRC000190	Dicofol	-5,415	-5,164		-5,671		-5,207	-5,583	
JRC000191	Diethylstilbestrol	-	-		-	-	-	-	
JRC000192	Estrone	-	-	-		-11,83	-	-	
JRC000193	Ethyl-4-hydroxybenzoate	-	-4,552		-4,826		-4,612	-5,107	
JRC000194	Fenarimol	-	-5,406		-5,376		-5,575	-5,506	

Validation requirements

- Purpose of the validation study needs to be clear.
- Availability of ‘reliable’ reference chemicals.
- Assay protocols have to be optimised and well defined.
- Appropriate and unambiguous acceptance criteria.
- Right data treatment and statistical analysis.
- Importance of transferability and reproducibility.
- Level of formality and independent peer-review.

One size doesn’t fit all !

Conclusions and outlook

- Automation is a powerful tool that can support and expedite the validation process is many ways.
- Nobody will be out of a job - some roles may change.
- Proactive education and communication on HTS methods are needed to reap the full benefit.
- You don’t have to have a HTS facility to contribute.
- Data is just data. Knowledge enables us to convert that data into useful information for safety assessment.

Acknowledgements

- research team at IHCP/ECVAM
- staff at NIEHS-NICEATM
- University of California Davis
- Tox21 consortium

Thank you !

http://ihcp.jrc.ec.europa.eu/

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