HIGH-THROUGHPUT PHENOTYPIC PROFILING OF 1470 TOXCAST CHEMICALS IN FIVE BIOLOGICALLY DIVERSE HUMAN-DERIVED CELL LINES USING THE CELL PAINTING ASSAY

Clinton Willis[1], Derik Haggard[1], Felix Harris[2], Gabrielle Byrd[2], Joshua Harrill[1]

[1]USEPA CCTE, RTP, NC [2]ORISE, Oak Ridge, TN

Clinton Willis | willis.clinton@epa.gov | 919-541-2849

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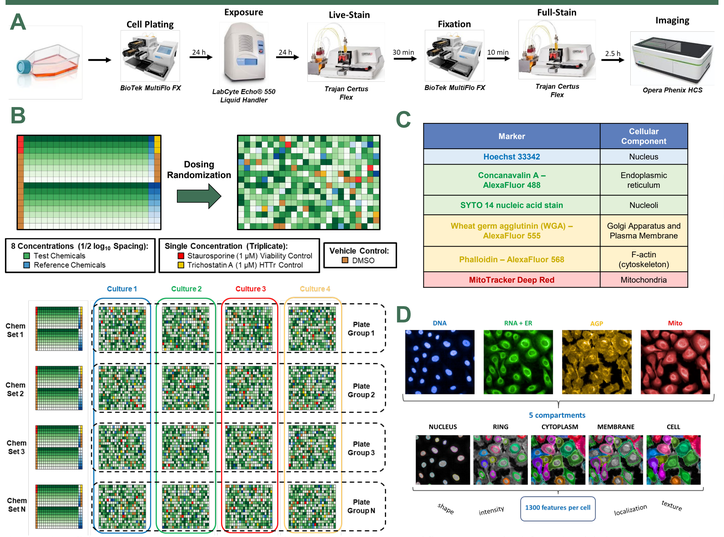
Background

- **High-Throughput Phenotypic Profiling (HTPP) with the Cell Painting** assay is a chemical screening method that combines high-content imaging and image analysis to measure phenotypic features at the single cell level and detect changes in cell morphology.
- HTPP has been proposed as a first-tier bioactivity screening assay for chemical hazard evaluation (Thomas et al. 2019, PMID: 30835285).
- This project aimed to screen **1,470 ToxCast chemicals** in a variety of human-derived cell lines and compare differences in response across cell lines.
- Cell lines were selected using a data-driven approach based on maximizing diversity of their baseline gene expression.
- **Phenotype Altering Concentrations (PACs)** are determined by concentration-response modeling and represent the threshold where changes in cell morphology begin to occur.

Cell Lines*	Tissue (Disease State)
HBEC3-KT	Lung; bronchial (hTERT Immortalized)
Ker-CT	Skin; foreskin (hTERT Immortalized)
MCF7	Breast; mammary gland (Cancer)
RPTEC/TERT-1	Kidney; proximal tube (hTERT Immortalized)
TeloHAEC	Heart; aorta (hTERT Immortalized)
ASC52Telo (Underway)	Adipose; mesenchymal stem cell (hTERT Immortalized)
U-2 OS (Planned)	Bone; tibia (Cancer)
CCD-18Co (Planned)	Colon; fibroblast (hTERT Immortalized)

*All cell lines acquired from American Type Culture Collection (ATCC)

Experimental Design



Marker	Cellular Component
Hoechst 33342	Nucleus
Concanavalin A – AlexaFluor 488	Endoplasmic reticulum
SYTO 14 nucleic acid stain	Nucleoli
Wheat germ agglutinin (WGA) – AlexaFluor 555	Golgi Apparatus and Plasma Membrane
Phalloidin – AlexaFluor 568	F-actin (cytoskeleton)
MitoTracker Deep Red	Mitochondria

Figure 1: Overall design for the study, including screening details, dose plate layout, and fluorescent marker information. (A) The HTPP assay utilizes a variety of microfluidic instruments that assist with plating, dosing, and staining plates prior to imaging. (B) All dose plates contain 42 test chemicals and 3 reference chemicals in 8-point concentration series, as well as additional vehicle and reference control treatments. Each dose plate is used to treat four independent cultures in randomized format using a Beckman Coulter Echo550® Liquid Handler 24h post-plating. (C) The table displays 6 different fluoroprobes used in the HTPP assay along with the organelles being labeled by each. (D) Fluoroprobe labeling patterns in four different imaging channels (shown in HBEC3-KT cells). Subsequent cell segmentation and image analysis are performed using PerkinElmer Harmony® software. These data are further analyzed using custom R scripts.

Results: Reference Chemicals Yield Reproducible Potencies

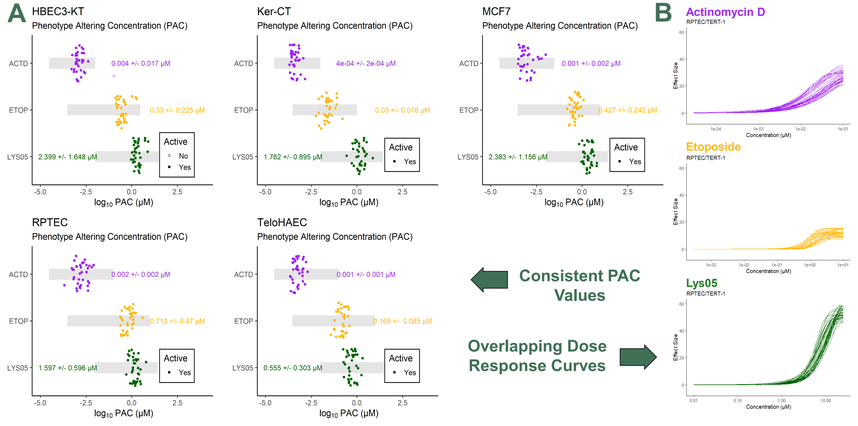


Figure 2: Phenotype altering concentrations (PACs) for 3 reference chemical shown across the 35 plate groups in each cell line tested. (A) Each individual plot corresponds to a cell line that was tested in this study and the 3 reference chemicals are listed on the *y*-axis. The gray band indicates the tested concentration range for that chemical with PAC values plotted on the *x*-axis. Mean and standard deviations for each condition are annotated on each plot. Each reference chemical was found to produce consistent PACs between plate groups for most cell lines. (B) Each concentration-response plot corresponds to a reference chemical with each curve modeling global Mahalanobis distance scores for each plate group. Results in RPTEC/TERT-1 cells are shown. Each plate group generated a reproducible concentration-response curve with similar effect sizes. ACTD = Actinomycin D, ETOP = Etoposide, LYS05 = Lys05 autophagy inhibitor.

Results: The Number of Active Chemicals Varies Across Cell Types

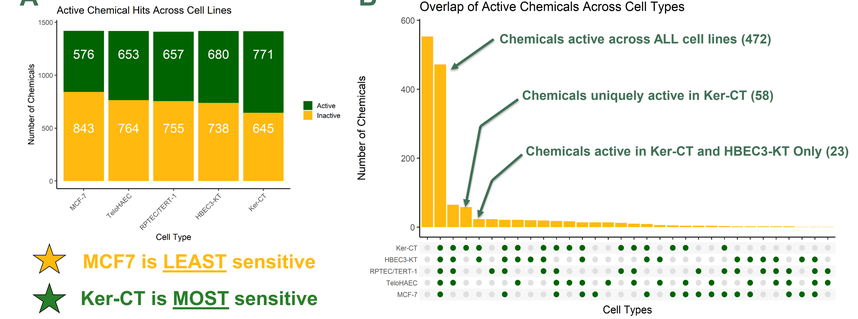


Figure 3: Examining the number of chemicals found to be active/inactive across the different cell lines. (A) The bar chart shows the number of chemicals that were determined to be active or inactive in each of the cell lines tested. MCF7 had the lowest number of active chemicals at 576 making it the least sensitive cell line. Ker-CT had the highest number of active chemicals at 771 making it the most sensitive cell line. (B) The upset plot shows the number of chemicals counted as active with the cell line combination highlighted on the *x*-axis. Chemicals inactive in all cell lines are tallied at the far left of the plot. Approximately 550 chemicals were found to be inactive in all cell lines and over 450 chemicals were found to be active in all 5 cell lines. 58 total chemicals were shown to be active in Ker-CT cells, only, and 23 chemicals active in Ker-CT and HBEC3-KT cells, only.

Results: Comparing Effect Size and Potency of Active Chemical Hits

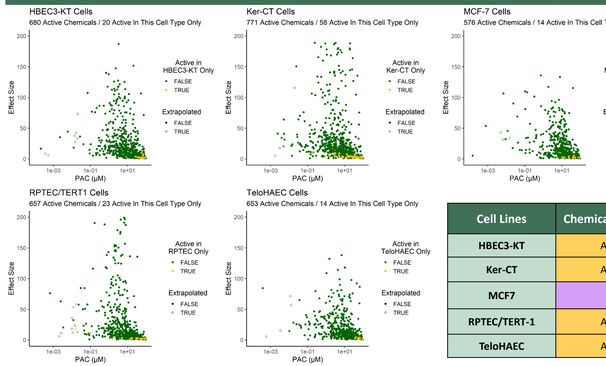


Figure 4: Examining PAC vs. effect size for active chemicals across cell types. The potency-magnitude plots represent *tcplfit2* concentration-response modeling of global Mahalanbois distance values for each cell line. Yellow points correspond to chemicals that were active in that cell type only. A majority of these chemicals had lower effect sizes and higher PAC values. Ker-CT had the most uniquely active chemical hits with MCF7 and TeloHAEC having the least. The chemical with the lowest PAC across all cell lines was Actinomycin D, except for MCF7 where Fulvestrant was the most potent. The chemical with the largest effect size was dependent on cell type.

Cell Lines	Chemical with Lowest PAC	Chemical with Largest Effect Size
HBEC3-KT	Actinomycin D	o,p'-DDE
Ker-CT	Actinomycin D	Naled
MCF7	Fulvestrant	1-Naphthylamine
RPTEC/TERT-1	Actinomycin D	Perfluorodecanoic acid
TeloHAEC	Actinomycin D	PharmaGSID_47337

Results: Comparing PACs Across Cell Types (Top 20 Chemicals)

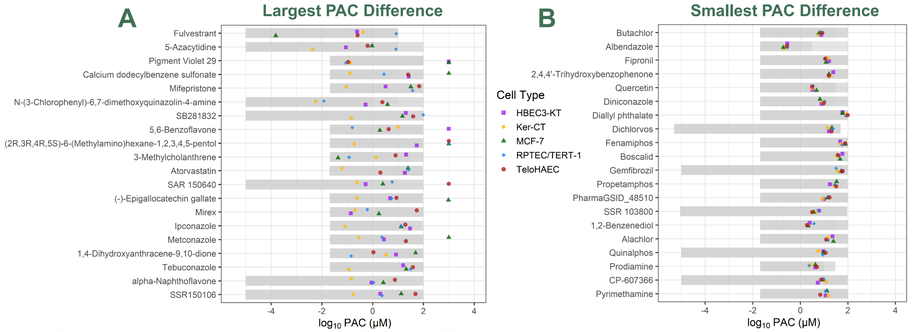


Figure 5: Potency rankings for the chemicals with the largest and smallest differences in PACs across cell types. (A) The 20 chemicals that had the largest PAC difference across cell types are listed on the *y*-axis going from top to bottom. PACs are plotted on the *x*-axis. The gray band indicates the range of tested concentrations, and each colored point represents a different cell type. Fulvestrant was found to have the biggest difference between PAC values across cell lines ranging from 1.5×10^{-4} µM [MCF7] to 8.49 µM [RPTEC/TERT-1]. The Ker-CT cell line is, again, shown to be the most sensitive with PAC values being lowest for a majority of chemicals in this list. (B) The 20 chemical that were active in all cell types and had the smallest PAC difference across cell types. The chemical with the most consistent PAC across all cell lines was butachlor with a PAC ranging from 5.57 µM [Ker-CT] to 8.34 µM [TeloHAEC].

Conclusions and Future Directions

- **Cell type is a determinant for chemical bioactivity in the HTPP assay.**
- **Next step: Perform high throughput toxicokinetic (HTTK) reverse dosimetry analysis. Compare results from different cell line combinations to in vivo toxicity values**