Assay Endpoint ID: 1825

ArunA_CellTiter_hNP

1. General Information

1.1 Assay Title: Viability Assessment in the ArunA Biomedical's Oris Neural Crest (hNC) Cell Migration Assay

1.2 Assay Summary: ArunA_CellTiter_hNP is a cell-based, single-readout assay that uses human H9-derived neuroprogenitor stem cells (hNP1).Measurements were taken 72 hours after chemical dosing in a 96-well plate. ArunA_CellTiter_hNP is an assay component measured from the ArunA_CellTiter_hNP assay. It is designed to make measurements of viability, a form of viability reporter, as detected with fluorescence intensity signals by HCS Fluorescent Imaging technology. Data from the assay component ArunA_CellTiter_hNP was analyzed at the endpoint, ArunA_CellTiter_hNP, in the positive analysis fitting direction relative to DMSO as the negative control and baseline of activity. Using a type of viability reporter, loss-of-signal activity can be used to understand the viability. To generalize the intended target to other relatable targets, this assay endpoint is annotated to the cell cycle intended target family, where the subfamily is cytotoxicity.

1.3 Date of Document Creation: September 05 2024

1.4 Authors and Contact Information:

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1.5 Assay Source: ArunA Biomedical is a privately owned biotechnology company and Contract Research Organization (CRO) formerly providing toxicology screening using neural stem cell-based assays.

1.6 Date of Assay Development: For date of assay development, see *Section 6: Bibliography*.

1.7 References: For complete list of references, see *Section 6: Bibliography*.

1.8 Proprietary Elements: This protocol describes the use of ArunA Biomedical's hNP1 Neural Progenitor Cells in conjunction with an Oris Cell Migration Assembly Kit- FLEX to measure the effect of neuroactive compounds and biologics that modulate proliferation and migration of neural progenitor cells. Certain uses of these products may be covered by U.S. Pat. No. 6,200,806; No. 7,531,354,B2 licensed to ARUNA and U.S. Pat. No. 7,842,499; No. 7,018,838; No. 10/597,118; No. 11/342,413; No. 11/890,740; and No. 12/195,007 licensed to PLATYPUS.

1.9 Assay Throughput: 96-well plate. ArunA systems offer high throughput chemical screening in a 96-well format for the human neuroprogenitor (hNP) and human neural crest (hNC) migration and cell titer endpoints.

1.10 Status: The assay is fully developed, and data are publicly available in ToxCast's invitroDB.

1.11 Abbreviations:

AIC: Akaike Information Criterion
AOP: Adverse Outcome Pathway
CV: Coefficient of Variation
DMSO: Dimethyl Sulfoxide
ToxCast: US EPA's Toxicity Forecaster Program
tcpl: ToxCast Data Analysis Pipeline R Package
SSMD: Strictly Standardized Mean Difference

2. Test Method Description

2.1 Purpose: Changes in fluorescence intensity related to the number of [3H]-thymidine labelled nuceli is indicative of the viability of the system.

Chemical-induced perturbations to cellular key events across neurogenic outcomes, including migration (neuroprogenitor and neural crest cells) and neural network formation (neurite length, neurite length, and branch points for neurites), in vitro can inform on cell-based prioritization of neurodevelopmental hazard potential.

2.2 Scientific Principles: During the development of the nervous systems, many processes occur to give rise to a functional and healthy neural network and hence nervous system. These important neurodevelopmental processes may be disrupted by potential toxicants, resulting in developmental neurotoxicity. Among these processes is migration of neuroprogenitors and neural crest cells (NCCs). Impaired neuroprogenitor and NCC

migration can lead to cerebral malformations and neurodevelopmental disorders, such as diencephalic-mesencephalic dysplasia syndrome, cerebral palsy, cerebellar ataxia, and microcephaly.

2.3 Experimental System: adherent hNP1 cell line used. The hN2 cell line is derived from neuroepithelial cells of WA09 hESC (Thomson et al., 1998) origin according to a previously described protocol (Shin et al., 2005, Shin et al., 2006). Importantly, as opposed to other methods of deriving neural progenitors through three-dimensional neurosphere and embryoid body formations (Reubinoff et al., 2001, Zhang et al., 2001), these adherent monolayer cultures are uniformly exposed to growth factors and/or morphogens throughout their propagation. Neurogenic lineages from human embryonic stem cell line WA09 were locked into three neural differentiation states: neuroprogenitor (hNP1 – Cat no. 7009), neural crest (hNC – Cat no. 7029), and neural network (hNN – Cat no. hNJL7014). Prior to differentiation into hN2 cells the population was confirmed karyotypically normal, >95% nestin positive and <3% OCT-4 positive (Shin et al., 2006). The cells were produced in bulk by propagation for an additional 2 weeks beyond the neuroepithelial stage by removal of bFGF from the media and cryopreserved (ArunA Biomedical, Athens, GA) for end user applications. The hNP and hNC cell endpoints consisted of cell titer and migratory measurements whereas hNN cell endpoints consisted of neuron count and three neurite-specific metrics to assess network formation: neurite length, neurites per neuron, and branch points for neurites. For this study, ArunA Bio extended the differentiation period of the hNN cells by approximately two weeks more than in the original hN2 protocol. This allowed for increased neural network cell axonation leading to better quantitation of network-specific endpoints. The utility of dissociated hN2 cultures as an in vitro model for neurite outgrowth was assessed using automated high-content image analysis (HCA). In addition, the molecular phenotype of these cells was examined using immunocytochemical staining.

2.4 Metabolic Competence: H9-derived cells are locked at different neuronal developmental states of interest to DNT investigations of chemical exposures. Xenobiotic biotransformation potential has not been characterized.

2.5 Exposure Regime: To assess the hNP and hNC migration and cell titer endpoints, 60,000 cells per well were plated onto Matrigel in basal growth medium with LIF and bFGF in a 96-well plate format. Plates were incubated for 16 h at 37C followed by a 72 h exposure to chemical in the test medium. For the migration endpoints, cells were seeded and incubated in presence of 'seeding stoppers' to prevent cell migration and growth into the detection zone. At the beginning of chemical exposure, stoppers were removed, and growth medium was replaced with test medium. In the case of the stopper control wells, stoppers remained in place following replacement of growth medium with test medium. Following 72 -h exposure to the test medium, cells were stained at 37C for 30−60 min with calcein-AM. Cell viability in the detection zone was quantitated using a Flexstation3 microplate reader (ex494 nm/em 517 nm). Corresponding cell titer endpoints were assessed for the hNP and hNC cells using the Promega CellTiter Aqueous One Solution Cell Proliferation Assay (Cat no. G3581; CellTiter 96). Finally, to gain insight into the mechanisms by which cells migrate into the detection zone, Ki-67 expression was quantified for 10 additional chemicals in the hNP and hNC systems. Additionally, cytochalasin D was used as a positive control to inhibit cell migration. Supplementing the AB2 Basal Medium: 1. Decontaminate the external surfaces of all supplement vials and the medium bottle with ethanol or isopropanol. 2. Aseptically open each supplement vial and add the amount indicated below to the basal medium with a pipette. To make 100 ml of complete medium: AB2 Neural Medium 96 mL, ANS Supplement 2 mL, bFGF (50 ug/mL) 40 uL, LIF (10 ug/mL) 100 uL, L-Glutamine (200 mM) 1 mL, Penicillin (5,000 U/mL)/ Streptomycin (5,000 µg/mL) 1 mL. 3. Supplemented medium should be stored at 2-8C, protected from light. The complete medium should be given a 2 week expiration date. Dispense the complete medium into aliquots to avoid repeated heating prior to each use. Plate Coating Protocol for hNP1 Neural Progenitor Expansion: To coat dishes perform the following steps: 1. Thaw BD Matrigel at 2-8C overnight. Matrix will gel rapidly at 22C to 35C. Keep Matrigel on ice and use pre-cooled pipettes, plates and tubes when preparing. Gelled Matrigel may be re-liquified if placed at 2-8C on ice for 24 to 48 hours. 2. Handle using aseptic technique in a laminar flow hood. 3. Once BD Matrigel Matrix is thawed, swirl vial to be sure that material is evenly dispersed. 4. Place thawed vial of BD Matrigel Matrix in sterile area, decontaminate the external surfaces with ethanol or isopropanol and air dry. BD Matrigel Matrix may be gently pipetted using a pre-cooled pipette to ensure homogeneity. 5. Dilute Matrigel 1:200 with cooled Dulbecco's Modified Eagle's Medium. Keep on ice. 6. Add 2 mL diluted Matrigel to a 35-mm dish. Swirl to ensure the entire surface of the 35-mm dish is covered with the Matrigel solution. 7. Place dishes

at 2-8C for 1-3 hours. 8. Rinse thoroughly with PBS. 9. Remove PBS and use immediately. Cell Thawing Protocol for hNP1 Neural Progenitor Expansion: To plate the cells perform the following steps: 1. Do not thaw the cells until the recommended medium and appropriately coated plasticware and/or glassware are on hand. 2. Remove the vial from liquid nitrogen and incubate in a 37C water bath. Closely monitor until the cells are completely thawed. Maximum cell viability is dependent on the rapid and complete thawing of frozen cells. IMPORTANT: Do not vortex the cells. Breaking cells down to single cell suspensions will significantly increase cell death. 3. As soon as the cells are completely thawed, disinfect the outside of the vial with 70% ethanol or isopropanol. Proceed immediately to the next step. 4. In a laminar flow hood, use a 1 or 2 mL pipette to transfer the cells to a sterile 15 mL conical tube. Be careful to not introduce any bubbles during the transfer process. 5. Using a 10 mL pipette, slowly add dropwise 9 mL of fully supplemented AB2 Neural Medium (pre-warmed to 37C) to the 15 mL conical tube. IMPORTANT: Do not add the whole volume of medium at once to the cells. This may result in decreased cell viability due to osmotic shock. 6. Gently mix the cell suspension by slow pipetting up and down twice. Be careful to not introduce any bubbles. IMPORTANT: Do not vortex the cells. Breaking cells down to single cell suspensions will significantly increase cell death. 7. Centrifuge the tube at room temperature at 200 x g for 4 minutes to pellet the cells. 8. Aspirate as much of the supernatant as possible. Steps 4-8 are necessary to remove residual cryopreservative (DMSO). 9. Resuspend the cells in a total volume of 2 mL of fully supplemented AB2 Neural Medium (pre-warmed to 37C). 10. Plate the 2 mL cell suspension of hNP1 cells onto a Matrigel-coated 35 mm dish. 11. Incubate the cells at 37C in a 5% CO2 humidified incubator. 12. Exchange the medium with fresh fully supplemented AB2 Neural Medium 24 hours post plating. Exchange with fresh medium every other day thereafter. Use caution not to dislodge the cells; do not pipette media directly onto the cells but rather onto the side of the culture dish. 13. Once the hNP1 cells reach 100% confluence, they can be dissociated manually for passaging (e.g., by cell scraping or by gentle and slow pipetting up and down to detach the cells). The cells should be maintained at a high density at all times – the recommended passaging ratio is 1:2. Subculture of hNP1 Cells: 1. Once the hNP1 cells reach 100% confluence, carefully remove the medium from the 35 mm dish. 2. Apply 2 mL fully supplemented AB2 Neural Medium (pre-warmed to 37C) to the cells so that the cells can be harvested in fresh medium. 3. Using a pipette, manually detach the cells from the dish by slow pipetting up and down the dish. Be careful to avoid introducing any bubbles. We recommend using a 200 uL or 1000 uL manual pipette to dislodge the attached cells. Alternatively, cells can be dislodged with a sterile cell scraper. IMPORTANT: We do NOT recommend enzymatic methods for passaging the hNP1 cells. Doing so reduces the long term viability of the cells and can cause karyotypic abnormalities. 4. Plates should be observed to ensure that all cells have been removed. This is most easily accomplished by working under a dissection microscope within a laminar flow hood, but can also be achieved by frequent observation under a bright field or phase contrast microscope. 5. Transfer the dissociated cells to a 50 mL conical tube. Inspect the plate to ensure that all the cells have been removed. 6. If necessary, count the cells and calculate the cell concentration. Cells can be centrifuged at 200 x g for 4 minutes in order to concentrate the cell suspension for higher plating densities. 7. Plate the cells at the desired density into the appropriately coated flasks, plates or wells in fully supplemented AB2 Neural Medium. We recommend keeping the cells at a high cell density by passaging 1:2. 8. Incubate the cells at 37C in a 5% CO2 humidified incubator. 9. Exchange the medium with fresh fully supplemented AB2 Neural Medium 24 hours post plating. Exchange with fresh medium every other day thereafter. Use caution not to dislodge the cells; do not pipette media directly onto the cells but rather onto the side of the culture dish. Plate Coating Protocol for Cell Migration Assay: 1. Thaw BD Matrigel at 2-8C overnight. Since it will gel rapidly at 22C to 35C, keep Matrigel on ice and use pre-cooled pipettes, plates and tubes when preparing. Gelled Matrigel may re-liquefy if placed at 2-8C on ice for 24 to 48 hours. 2. Handle using aseptic technique in a laminar flow hood. 3. Once the Matrigel is thawed, swirl vial to be sure that material is evenly dispersed. 4. Place thawed vial of Matrigel in sterile area, decontaminate the external surfaces with ethanol or isopropanol and air dry. Matrigel may be gently pipetted using a pre-cooled pipette to ensure homogeneity. 5. Dilute Matrigel 1:200 with cooled AB2 Neural Culture Medium. Prepare 1 mL diluted Matrigel for each column (8 wells) to be used. Keep on ice. 6. Add 100 uL of diluted Matrigel to each well intended for use in the 96 well plate. 7. Tap the plate gently to ensure the entire surface of the well is covered with diluted Matrigel. 8. Place dishes at 2-8C for 1-3 hours. 9. Remove the residual coating solution and rinse each well twice with 200 uL of PBS per well. 10. Remove PBS and insert the Oris Cell Seeding Stoppers into the coated wells of the 96-well plate. 11. Visually inspect to ensure that the Oris Cell Seeding Stoppers are firmly sealed. Cell Migration Assay Protocol: 1. Harvest cells as described in steps 1-5 of section Subculture of hNP1 Neural

Progenitor cells. 2. Count cells and adjust cell suspension volume to the following concentration: 600,000 cells/mL 3. Plate 100 uL of suspended cells into each stoppered well for a cell density of 60,000 cells per well. 4. Incubate the cells at 37C in a 5% CO2 humidified incubator overnight (16-24 hours) to permit cell attachment. 5. Using the Oris Stopper Tool, remove all stoppers, except for those in "no migration controls" which will remain in place until time of staining. 6. Carefully remove the seeding media from the wells and add 200 uL medium containing the test compound per well. 7. Briefly examine the wells by phase contrast microscopy to ensure continued adherence of the cells. 8. Incubate the cells at 37C/5% CO2 for 72 hours to permit cell migration. 9. After 72 hours, mix 5 uL Calcein AM, 5 uL Hoechst 33342, and 10 mL phenol red-free Neurobasal medium with 0.1% BSA. 10. Carefully remove stoppers from the “no migration controls”. 11. Carefully remove the test medium from all wells and add 100 µL of diluted Calcein/Hoechst solution to each well. 12. Incubate plate at 37C/5% CO2 for 30 - 60 minutes with the lid on and in the dark (the darkness of a standard incubator will suffice). 13. For use with a fluorescence microplate reader, attach the Oris Detection Mask and read promptly for Calcein fluorescence (ex 494 nm/ em 517 nm). 14. For image analysis, photomicrograph wells using epifluorescence illumination with or without the Oris Detection mask. Images can then be analyzed using either area closure with the calcein stain or number of cells (nuclei) using the Hoechst stain. ImageJ freeware available from the NIH (http://rsbweb.nih.gov/ij/) can be used for migration data analysis as percent area closure or cellular enumeration

ASSAY DESIGN SUMMARY	
Nominal number of tested concentrations: 5	Target (nominal) number of replicates: 4
Standard minimum concentration tested: 1.2 µM	Standard maximum concentration tested: 100 µM
Key positive control: NA	Neutral vehicle control: DMSO
Baseline median absolute deviation for the assay (bmad): 0.115	
Response cutoff threshold used to determine hit calls: 0.344	
Detection technology used: HCS Fluorescent Imaging (Fluorescence)	

2.6 Response: The ArunA migration assay measures growth and survival in human embryonic neuroprogenitor (hNP) and human neural crest (hNC) cells by tracking the presence/absence of viable nuclei movement into a defined circular area in each microplate well. These different measurements are assessed following 72 hour incubations with test chemical to evaluate the potential to disrupt neural migration in developing human embryos.

2.7 Quality and Acceptance Criteria: Each assay may utilize different acceptance criteria and quality assurance methods as it pertains to the individual assay platform and implementation. Pre-processing transformations may indicate issues in plates or wells by setting well quality (wllq) values to 0. Analytical QC calls per sample and substance can also be considered to understand the applicability domain of the chemicals for screening.

2.8 Technical Limitations: ToxCast data can provide initial (screening) information about the capacity for a chemical to illicit a biological response; caution is advised with extrapolation of these results to organism-level responses. The potential for a chemical to elicit adverse health outcomes in living systems is a function of multiple factors, and this assay is not intended to provide predictive details regarding long term or indirect adverse effects in complex biological systems but can aid in the prioritization of compound selection for more resource intensive toxicity studies. See *Section 4.4*. for more information on the chemical applicability of the assay.

2.9 Related Assays: For related assays, consult the following assay lists or intended target families. This assay is present in the following assay lists:

NA

Additionally, this assay was annotated to the intended target family of cell cycle.

3. Data Interpretation

The ToxCast Data Analysis Pipeline (tcpl) R package includes processing functionality for two screening paradigms: (1) single-concentration ("SC") and (2) multiple-concentration ("MC") screening. SC screening consists of testing chemicals at one concentration, often for the purpose of identifying potentially active chemicals to test in the multiple-concentration format. MC screening consists of testing chemicals across a concentration range, such that the modeled activity can give an estimate of potency, efficacy, etc. MC data is the focus of this documentation, with SC data processing metrics to be incorporated in the future.

3.1 Responses captured in prediction model: See *Section 2.6* for additional information on responses measured.

3.2 Data Analysis: The migration of neuroprogenitor and neural crest cells into the detection zone was assessed by comparing the percent migration of proliferative Ki-67 cells to total migrating cells following 72 hours exposure. This was accomplished by determining the percentage of total cells migrating into the detection zone, i.e. the migration index (MI), compared to the percentage of migrating cells that expressed the Ki-67 proliferative marker within the detection zone, i.e. the proliferative index (PI). Normalized response values for each assay endpoint were calculated as resp = 100 x (rval-bval) / (pval-bval) where rval, bval, and pval correspond to the raw value, the plate level DMSO control median, and the plate level positive/negative control median, respectively. In the parallel viability assessment, normalized response was calculated as resp = log2(rval/bval).

Prior to the data processing, all the data must go through pre-processing to transform the heterogeneous data into a uniform format before it can be loaded into a database. Level 0 pre-processing is done in R by vendor/dataset-specific scripts with all manual transformations to the data documented with justification. Common examples of manual transformations include fixing a sample ID typo or changing well quality value(wllq) to 0 after identifying problems such a plate row/column missing an assay reagent.

Once data is loaded into the database, tcpl utilizes generalized processing functions provided to process, normalize, model, qualify, and visualize the data. To promote reproducibility, all method assignments must occur through the database and should come from the available list of methods for each processing level. Assigned multiple concentration processing methods include:

Level 2: Component-specific corrections include:

1: none (Use corrected response value (cval) as is; cval = cval. No additional mc2 methods needed for component-specific corrections.)

Level 3: Endpoint-specific normalization include:

7: resp.log2 (Transform the response values to log-scale (base 2).), 9: resp.fc (Calculate the normalized response (resp) as the fold change, i.e. the ratio of the corrected (cval) and baseline (bval) values; resp = cval/bal.), 11: bval.apid.nwlls.med (Calculate the baseline value (bval) as the plate-wise median, by assay plate ID (apid), of the corrected values (cval) for neutral control wells (wllt = n).)

Level 4: Baseline and required tcplFit2 parameters defined by:

2: bmad.aeid.lowconc.nwells (Calculate the baseline median absolute value (bmad) as the median absolute deviation of normalized response values (resp) for neutral control wells (wllt = n). Calculate one standard deviation of the normalized response for neutral control wells (wllt = n); onesd = sqrt(sum((resp - mean resp)^2)/sample size - 1). Onesd is used to establish BMR and therefore required for tcplfit2 processing.)

Level 5: Possible cutoff thresholds, where higher value for endpoint is selected, include:

1: bmad3 (Add a cutoff value of 3 multiplied by the baseline median absolute deviation (bmad) as defined at Level 4.), 27: ow_bidirectional_loss (Multiply winning model hitcall (hitc) by -1 for models fit in the positive analysis direction. Typically used for endpoints where only negative responses are biologically relevant.)

Level 6: Cautionary flagging include:

5: modl.directionality.fail (Flag series if model directionality is questionable, i.e. if the winning model direction was opposite, more responses (resp) would have exceeded the cutoff (coff). If loss was winning directionality (top < 0), flag if count(resp < -1*coff) < 2*count(resp > coff). If gain was winning directionality (top > 0), flag if count(resp > coff) < 2*count(resp < -1*coff).), 6: singlept.hit.high (Flag single-point hit that's only at the highest conc tested, where series is an active hit call (hitc >= 0.9) with the median response observed above baseline occurring only at the highest tested concentration tested.

), 7: singlept.hit.mid (Flag single-point hit that's not at the highest conc tested, where series is an active hit call (hitc >= 0.9) with the median response observed above baseline occurring only at one concentration and not the highest concentration tested.), 8: multipoint.neg (Flag multi-point miss, where series is an inactive hit call (hitc < 0.9) with multiple median responses observed above baseline.), 9: bmd.high (Flag series if modeled benchmark dose (BMD) is greater than AC50 (concentration at 50 percent maximal response). This is indicates high variability in baseline response in excess of more than half of the maximal response.), 10: noise (Flag series as noisy if the quality of fit as calculated by the root mean square error (rmse) for the series is greater than the cutoff (coff); rmse > coff.), 11: border (Flag series if borderline activity is suspected based on modeled top parameter (top) relative to cutoff (coff); |top| <= 1.2(coff) or |top| >= 0.8(coff).), 13: low.nrep (Flag series if the average number of replicates per concentration is less than 2; nrep < 2.), 14: low.nconc (Flag series if 4 concentrations or less were tested; nconc <= 4.), 15: gnls.lowconc (Flag series where winning model is gain-loss (gnls) and the gain AC50 is less than the minimum tested concentration, and the loss AC50 is less than the mean tested concentration.), 17: efficacy.50 (Flag low efficacy hits if series has an active hit call (hitc >= 0.9) and efficacy values (e.g. top and maximum median response) less than 50 percent; intended for biochemical assays. If hitc >= 0.9 and coff >= 5, then flag when top < 50 or max_med < 50. If hitc >= 0.9 and coff < 5, then flag when top < log2(1.5) or max_med < log2(1.5).), 18: ac50.lowconc (Flag series with an active hit call (hitc >= 0.9) if AC50 (concentration at 50 percent maximal response) is less than the lowest concentration tested;if hitc >= 0.9 and ac50 < 10^logc_min, then flag.), 19: viability.gnls (Flag series with an active hit call (hitc >= 0.9) if denoted as cell viability assay with winning model is gain-loss (gnls); if hitc >= 0.9, modl=="gnls" and cell_viability_assay == 1, then flag.), 20: no.med.gt.3bmad (Flag series where no median response values are greater than baseline as defined by 3 times the baseline median absolute deviation (bmad); nmed_gtbl_pos and nmed_gtbl_neg both = 0, where nmed_gtbl_pos/_neg is the number of medians greater than 3*bmad/less than -3*bmad.)

The following is an aggregate endpoint summary of the number of samples and chemicals tested, as well as active or inactive hit calls (hitc) and predicted winning models for all samples tested in this endpoint.

SAMPLE AND CHEMICAL COVERAGE		
Number of samples tested: 63		Number of chemicals tested: 58
ACTIVITY HIT CALLS		
Active hit count: hitc≥0.9 20	Inactive hit count: 0≤hitc<0.9 21	NA hit count: *hitc<0* *22*

WINING MODEL SELECTION	
Number of sample-assay endpoints with winning *hill* model:	5
gain-loss (gnls) model:	12
power(pow) model:	4
linear-polynomial (poly1) model:	6
quadratic-polynomial(poly2) model:	10
exponential-2 (exp2) model:	1
exponential-3 (exp3) model:	0
exponential-4 (exp4) model:	18
exponential-5 (exp5) model:	7

For each concentration series, several point-of-departure (POD) estimates are calculated for the winning model. The major estimates include: (1) the activity concentration at the specified benchmark response (BMR) (bmd),

(2) the activity concentration at 50% of the maximal response (ac50), (3) the activity concentration at the efficacy cutoff (acc), (4) the activity concentration at 10% of the maximal response, and (5) the concentration at 5% of the maximal response.

3.3 Prediction Model: All statistical analyses were conducted using R programming language, employing the tcpl package to generate model parameters and confidence intervals. Each chemical concentration response series is fit to ten predictive models, encoded by the dependency package tcplfit2. The models include the constant, Hill, gain-loss, two polynomials (i.e. linear and quadratic), power, and four exponential variants. The polynomials, power, and exponential models are all based on BMDExpress2. The winning model (modl) is selected based on the lowest AIC value and is used to determine the activity (or hit call) for the concentration series. If two models have equal AIC values, then the simpler model (i.e. model with fewer parameters) wins. In invitrodb, levels 4 and 5 capture model fit information. mc4 captures summary values calculated for each concentration series, whereas mc4_param stores the estimated model parameters for all models fit to data in long format. mc5 captures the winning model selected and the activity hit call, whereas mc5_param stores the estimated model parameters for the selected winning model in long format. Activity for each concentration-response series is determined by calculating a continuous hit-call for the winning model, which is the product of three proportional weights. The first weight reflects whether there is at least one median response outside the efficacy cutoff band. Second, the top (or maximal change in the predicted response) is larger than the cutoff. The last weight reflects whether the AIC of the winning model is less than the constant model, i.e. the winning model is better fit than a flat line.

The continuous hit call value (hitc), fit category (fitc), and cautionary flags (mc6) can be used to understand the goodness-of-fit, enabling the user to decide the stringency with which to filter and interpret results. Hitc may be further binarized into active or inactive, depending on the level of stringency required by the user; herein, hitc greater than or equal to 0.90 is active, hitc between 0 and 0.90 is inactive, and hitc less than 0 is not applicable, but different thresholds may be used. Fitc was summarize curve behavior relative activity, efficacy, and potency comparisons between the AC50 and the concentration range screened. Cautionary flags on fitting were developed in previous versions of tcpl and have been stored at level 6. These flags are programmatically generated and indicate characteristics of a curve that need extra attention or potential anomalies in the curve or data. Users may review these filtered groupings to understand high-confidence curves.

3.4 Software: The ToxCast Data Analysis Pipeline (tcpl) is an R package that manages, curve-fits, plots, and stores ToxCast data to populate its linked MySQL database, invitrodb. Data for invitrodb v4.2 was processed using the tcpl v3.2. See *Section 7: Supporting Information* on the ToxCast program and tcpl R package.

4. Test Method Performance

4.1 Robustness: The following assay performance metrics surmise the robustness of the method i.e. the reliability of the experimental results and the prediction capability of the model used.

NEUTRAL CONTROL (well type = "n")	
Neutral control well median response value, by plate: *nmed*	1.332
Neutral control median absolute deviation, by plate: *nmad*	0.103
Coefficient of variation (CV%) in neutral control wells: *(nmad/nmed)*100*	7.79%
POSITIVE CONTROL (well type = "p")	
Positive control well median response value, by plate: *pmed*	NA
Positive control well median absolute deviation, by plate: *pmad*	NA
Z Prime Factor for median positive and neutral control across all plates: *(1 - ((3 * (pmad + nmad)) / abs(pmed - nmed))*	NA
Strictly standardized mean difference (SSMD) for positive compared to neutral control wells: *((pmed - nmed) / sqrt($pmad^2$ + $nmad^2$)*	NA

Positive control signal-to-noise: *((pmed-nmed)/nmad)*	NA
Positive control signal-to-background: *(pmed/nmed)*	NA
NEGATIVE CONTROL (well type = "m")	
Negative control well median, by plate: *mmed*	NA
Negative control well median absolute deviation value, by plate: *mmad*	NA
Z Prime Factor for median negative and neutral control across all plates: *(1 - ((3 * (mmad + nmad)) / abs(mmed - nmed))*	NA
Strictly standardized mean difference (SSMD) for negative compared to neutral control wells: *((mmed - nmed) / sqrt($mmad^2$ + $nmad^2$)*	NA
Signal-to-noise (median across all plates, using negative control wells): *((mmed-nmed)/nmad)*	NA
Signal-to-background (median across all plates, using negative control wells): *(mmed/nmed)*	NA

4.2 Reference Chemical Information: Reference chemical curation is ongoing, and this section will be updated as more information becomes available.

4.3 Performance Measures and Predictive Capacity: The performance and predictivity for a given assay may be evaluated with a variety of performance statistics but is dependent upon available data. Predictive capacity (i.e. false negative, false positive rates) will be assessed when reference chemical information is available. Ideally, assays will have sufficient data on reference chemicals (i.e. positive and negative controls) to enable estimation of accuracy statistics, such as sensitivity and specificity.

ToxCast targets may align to a range of event types in the Adverse Outcome Pathway (AOP) framework, however each assay technology may have specific limitations, which may require user discretion for more complex interpretations of the data.

The median root mean squared error (RMSE) across all winning models for active hits was calculated as: 7.

4.4 Chemical Library Scope and Limitations: The ToxCast Chemical Library was designed to capture a large spectrum of structurally and physicochemically diverse compounds. This chemical inventory incorporates toxicity data-rich chemicals, chemicals spanning major use-categories, and chemicals with exposure potential, including but not limited to pesticides, antimicrobials, fragrances, green chemistry alternatives, food additives, toxicity reference compounds and failed pharmaceuticals. In addition to environmental or exposure concerns, chemical selection criteria also consider practical constraints, such as commercial availability, dimethyl sulfoxide (DMSO) solubility and stability, and suitability for testing in automated or semi-automated systems (e.g., low volatility and moderate LogP values). Under these constraints, there were three major, interrelated drivers for chemical selection: availability of animal toxicity data or mechanistic knowledge, exposure potential, and EPA regulatory interest. The first driver would provide the necessary *in vivo* and mechanistic data to anchor and validate subsequent prediction modeling efforts, whereas the latter two were intended to provide coverage of the chemical landscape to which humans and ecosystems are potentially exposed and for which toxicity data are mostly lacking. Analytical QC calls per sample and substance should be considered to understand the applicability domain of the chemicals for screening.

5. Potential Regulatory Applications

5.1 Context of Use: Examples of end use scenarios could include, but are not limited to:

- *Support Category Formation and Read-Across:* The outcomes from the assay could be used to substantiate a hypothesis for grouping substances together for the purposes of read-across,
- *Priority Setting:* The assay might help prioritize substances within an inventory for more detailed evaluation,

- *Screening Level Assessment of a Biomarker or Mechanistic Activity or Response:* The screening level assessment may be sufficient to identify a hazard and provide a gauge of potency; or
- *Integrated approaches to testing and assessment (IATA):* The assay may form one component of an IATA.

6. **Bibliography:** Zurlinden TJ, Saili KS, Baker NC, Toimela T, Heinonen T, Knudsen TB. A cross-platform approach to characterize and screen potential neurovascular unit toxicants. Reprod Toxicol. 2020 Jun 24;96:300-315. doi: 10.1016/j.reprotox.2020.06.010. Epub ahead of print. PMID: 32590145.

7. **Supporting Information:**
More information on the ToxCast program can be found at: https://www.epa.gov/chemical-research/toxicity-forecasting. The most recent version of downloadable data can be found at: https://www.epa.gov/chemical-research/exploring-toxcast-data-downloadable-data. The ToxCast Data Analysis Pipeline (tcpl) R package is available on CRAN or GitHub. Check out tcpl's vignette for comprehensive documentation describing ToxCast data processing, retrieval, and interpretation.