Assay Endpoint ID: 1816

TOX21_AR_LUC_MDAKB2_Antagonist_0.5nM_R1881

1. General Information

1.1 Assay Title: Tox21 MDa-kb2 Androgen Receptor (AR) Antagonism (0.5nM R1881) Luciferase Assay

1.2 Assay Summary: TOX21_AR_LUC_MDAKB2_Antagonist_0.5nM_R1881 is a cell-based, single-readout assay that uses MDA-kb2, a human breast cell line, with measurements taken at 24 hours after chemical dosing in a 1536-well plate. See tox21-ar-mda-kb2-luc-antagonist-p2. TOX21_AR_LUC_MDAKB2_Antagonist_0.5nM_R1881 is one of one assay component(s) measured or calculated from the TOX21_AR_LUC_MDAKB2_Antagonist_0.5nM_R1881. It is designed to make measurements of luciferase induction, a form of inducible reporter, as detected with bioluminescence signals following addition of luciferin substrate and ATP. Data from the assay component TOX21_AR_LUC_MDAKB2_Antagonist_0.5nM_R1881 was analyzed into 1 assay endpoint. This assay endpoint, TOX21_AR_LUC_MDAKB2_Antagonist_0.5nM_R1881, was analyzed in the positive analysis fitting direction relative to DMSO as the negative control and baseline of activity. Using a type of inducible reporter, loss-of-signal activity can be used to understand changes in the reporter gene as they relate to the gene AR. Furthermore, this assay endpoint can be referred to as a primary readout, because the performed assay has only produced 1 assay endpoint. To generalize the intended target to other relatable targets, this assay endpoint is annotated to the nuclear receptor intended target family, where the subfamily is steroidal.

1.3 Date of Document Creation: September 05 2024

1.4 Authors and Contact Information:

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1.5 Assay Source: Tox21 is an interagency agreement between the NIH, NTP, FDA and EPA. NIH Chemical Genomics Center (NCGC) is the primary screening facility running ultra high-throughput screening assays across a large interagency-developed chemical library.

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 assay is not proprietary. The Tox21 qHTS robotic platform has a 1536-well per run capacity and is capable of fully-automated, hands-free execution (liquid dispensing and aspiration, plate centrifugation and incubation) and signal recording (plate readout). The GeneBLAzer System is publically available through Invitrogen.

1.9 Assay Throughput: 1536-well plate. Stably transfected MDA-kb2 cells are aliquoted into 1536-well microtiter plates and incubated with test compounds for 16 hours prior to monitoring luminescence resulting from AR gene expression.

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: Androgen receptor (AR) is an important member of the nuclear receptor family. Its signaling plays a critical role in AR-dependent prostate cancer and other androgen related diseases. Considerable attention has been given in the past decades to develop methods to study and screen for the environmental chemicals that have the potential to interfere with metabolic homeostasis, reproduction, developmental and behavioral functions. Changes to bioluminescence signals produced from an enzymatic reaction involving the key substrate

[One-Glo] are indicative of changes in transcriptional gene expression due to antagonist activity regulated by the human androgen receptor [GeneSymbol:AR | GeneID:367 | Uniprot_SwissProt_Accession:P10275].

The Tox21 androgen receptor antagonism luciferase assay screened a large library of diverse environmental compounds to probe for xenobiotic androgenic activity and potential to inhibit androgen-dependent transcription, monitored through luciferase reporter gene signal activity using an AR-luciferase reporter gene construct. The assay is run in triplicate on a 1536-well microplate and bioluminescence was measured following 15.5 hour incubation of cells with test compounds and 30 min incubation of test system with ONE-GloTM luciferase assay reagent. The bioluminescent signal was monitored using a Promega ViewLux plate reader. Following the incubation period, the cell culture was screened for bioluminescent signals in antagonist mode using luciferase detection technology. Each compound was tested in a concentration-response format, using 15 concentrations ranging from 1.1 nM to 92 uM. To help distinguish true antagonistic activity from cytotoxic effects, this assay was multiplexed with a fluorescence-based cell viability assay which measured conserved and constitutive protease activity within live cells (Promega). Compound auto-fluorescence was monitored in various "TOX21_AutoFluor" assays run at interfering wavelengths to allow for background artifact detection. Concentration-response relationships were determined by monitoring luminescent signals relative to DMSO-only exposures which provided a signal baseline, and to a known androgen receptor antagonist (Nilutamide) as a positive control which provided a reference for 100 percent androgen receptor inhibition, as assessed in the presence of 0.5 uM R1881, a known AR agonist.

2.2 Scientific Principles: Luciferase reporter-gene assays are a commonly used bioluminescence assay. The construct includes a promoter region of a gene of interest followed by a luciferase gene. When this is introduced into a cell, luciferase is expressed in quantities that are proportional to the promoter activity. The luciferase (and hence the promoter activity) can then be quantified by the measurement of the luminescence produced when the enzyme substrate is added. In this way, the transcriptional activity of the gene of interest (i.e., its expression) can be measured in response to the effects of different modulators of the relevant signaling pathways. The luciferase reaction can also be used in combination with constitutively active promoters, to investigate cytotoxicity or transfection efficiency. Androgen receptors have pleiotropic regulatory roles in a diverse range of tissues; particularly in mediating the activity of endogenous androgens in the hypothalamus, pituitary, liver, prostate, and testicular tissues. Endogenous androgens are important for target gene expression in physiological processes like developmental differentiation of the male embryo, initiation and maintenance of spermatogenesis and in neuroendocrine system functioning. Endogenous androgens also influence male pubertal maturation and maintenance of secondary sexual characteristics in adults. Disruption of androgen signaling following exposure to endocrine-mimicking environmental chemicals can result in hormonal cancers, impaired reproductive development and infertility. This assay is designed to screen a large, structurally diverse chemical library to identify compounds capable of interference with endogenous androgenic signaling by monitoring the increase in luminescent signals relative to a known androgen receptor agonist (Methyltrienolone) as a positive control, and indicator of androgenic activity. The Tox21 MDA-kb2 AR luciferase assays are qHTS format assays which measured the ability of a chemical to interact with AR by monitoring modulation of luminescent reporter gene signals. This assay utilized an epithelial breast cancer cell line which expresses firefly luciferase under control of a MMTV promoter that contains androgen response elements to quantify xenobiotic androgen receptor agonism. This assay is intended for use as a part of an integrated testing strategy, to screen a large structurally diverse chemical library for compounds with the potential to interact with androgen receptor mediated pathways and potentially affect endocrine systems in exposed populations. There is strong evidence that androgen receptor agonism is a molecular initiating event (MIE) in an Adverse Outcome Pathway (AOP) leading to reproductive dysfunction in fish populations (EAGMST Approved AOP), and there is some evidence that androgen receptor activation is the MIE for a putative pathway leading to hepatocellular adenomas and carcinomas (in mouse and rat models) (AOP currently under development). Chemical-activity profiles derived from this assay can inform prioritization decisions for compound selection in more resource intensive in vivo studies to further investigate the involvement of AR agonism in pathways leading to hazardous outcomes in biological systems.

2.3 Experimental System: adherent MDA-kb2 cell line used. The MDA-kb2 AR-luc cell line was derived from epithelial breast cancer cell line, MDA-MB-453 (originally obtained in 1976 from pleural effusion of metastatic carcinoma from 48-yo Caucasian female) by stable transfection with a mouse mammary tumor virus (MMTV) neomycin-resistant luciferase reporter gene construct. MDA-MB-453 cells have fibroblastic morphology and were selected for transformation due to high levels of functional, endogenous androgen and glucocorticoid receptors while estrogen receptor (ER) alpha and progesterone receptor (PR) mRNA are not detectable, and ER-beta is apparently expressed at very low levels. This cell line expresses firefly luciferase under control of a MMTV promoter that contains response elements for both GR and AR. MDA-kb2 may be used in an in vitro assay to screen androgen agonist and antagonists and to characterize its specificity and sensitivity to endocrine disrupting chemicals.

2.4 Metabolic Competence: Xenobiotic biotransformation potential has not been characterized. Metabolic activity has been examined for the parental MDA-MB-453 cells. CYP1A1 and CYP1B1 have been shown to be inducible following TCDD exposures, with exposure to AhR agonists showing highly preferential induction of CYP1B1 as opposed to CYP1A1.

2.5 Exposure Regime: Quality Control Precautions: Maintain cell culture below 85-90% confluence. Cell culturing and assay culture doesn't require CO2. Cell Thawing Method: Thaw a vial of cells in 9ml of pre-warmed thaw/culture medium and then centrifuge. Resuspend the pellet with the thaw/culture medium and seed at 2 million cells per T-75 flask. Cell Proliferation Method: Trypsinize cells from the culturing flask and centrifuge and then resuspend cells in culture medium. Passage cells at 6-7 million per T-225 flask. Assay Protocol: Trypsinize cells from the culturing flask and centrifuge and then resuspend cells in culture/assay medium. Dispense 3000 cells/5uL/well (for agonist mode) into 1536-well tissue treated white/solid bottom plates using a 8 tip dispenser (Multidrop). Incubate the assay plates for 5-6hrs at 37C and 0% CO2. Transfer 23nL of compounds from the library collection and positive control/DMSO into the assay plates through Pintool. Compound transfer was followed by the addition of 1uL of 0.5nM (final concentration) R1881 or assay buffer using 2 tips of a dispenser (BioRAPTR FRD). Incubate the assay plates for 16hrs at 37C and 0% CO2. After 15hrs of incubation at 37C and 0%CO2, 1ul of CellTiter-Fluor reagent was added using a single tip of a dispenser (BioRAPTR FRD). Incubate the assay plates at 37C and 0%CO2 for 1hr. Read fluorescence using ViewLux plate reader. Then followed by the addition of 4ul of ONE-Glo(TM) Luciferase reagent using a single tip of a dispenser (BioRAPTR FRD). Incubate the assay plates at room temperature for 30min. Read Luminescence using ViewLux plate reader.

ASSAY DESIGN SUMMARY	
Nominal number of tested concentrations: 15	Target (nominal) number of replicates: 3
Standard minimum concentration tested: 0.0117795391705069 µM	Standard maximum concentration tested: 920.276497695853 µM
Key positive control: Nilutamide	Neutral vehicle control: DMSO
Baseline median absolute deviation for the assay (bmad): 5.354	
Response cutoff threshold used to determine hit calls: 32.127	
Detection technology used: CellTiter-Glo Luciferase-coupled ATP quantitation (Luminescence)	

2.6 Response: Androgen receptor antagonism and inhibited gene expression is measured by monitoring luminescent production by the luciferase reporter gene under control of androgen response element promoters. The cytotoxicity of the compounds screened was measured in parallel by measuring the cell viability using by CellTiter-Glo Luciferase-coupled ATP quantitation in the same wells.

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:

ToxCast AR Pathway Model: Androgen receptor assays used in ToxCast AR Pathway model. See 10.1016/j.yrtph.2020.104764 and 10.1021/acs.chemrestox.6b00347

Additionally, this assay was annotated to the intended target family of nuclear receptor.

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: In cultures stimulated with a known agonist (0.5nM R1881), decreased luminescence (loss-of-signal) relative to nilutamide signal (positive control, 100 percent antagonist activity), using DMSO (neutral control) as a signal baseline as a baseline for luciferase induction. Response was reported as a percent of positive control activity. This is an secondary assay to TOX21_AR_LUC_MDAKB2_Agonist to confirm antagonist specificity.

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:

1: none (Set the corrected response value (cval) as the normalized response value (resp); cval = resp. No additional mc3 methods needed for endpoint-specific normalization.)

Level 4: Baseline and required tcplFit2 parameters defined by:

1: bmad.aeid.lowconc.twells (Calculate the baseline median absolute value (bmad) as the median absolute deviation of normalized response values (rep) for test compound wells (wllt = t) with concentration index (cndx) equal to 1 or 2. Calculate one standard deviation of the normalized response for test compound wells (wllt = t) with a concentration index (cndx) of 1 or 2; 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:

2: pc20 (Add a cutoff value of 20. Typically for percent of control data.), 6: bmad6 (Add a cutoff value of 6 multiplied by the baseline median absolute deviation (bmad). By default, bmad is calculated using test compound wells (wllt = t) for the endpoint.), 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.), 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: 9667		Number of chemicals tested: 7871
ACTIVITY HIT CALLS		
Active hit count: hitc≥0.9 1763	Inactive hit count: 0≤hitc<0.9 4681	NA hit count: *hitc<0* *3223*
WINING MODEL SELECTION		
Number of sample-assay endpoints with winning *hill* model:	789	
gain-loss (gnls) model:	536	
power(pow) model:	715	
linear-polynomial (poly1) model:	3114	

	quadratic-polynomial(poly2) model:	1100
	exponential-2 (exp2) model:	295
	exponential-3 (exp3) model:	42
	exponential-4 (exp4) model:	2231
	exponential-5 (exp5) model:	845

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*	0
Neutral control median absolute deviation, by plate: *nmad*	8.677

Metric	Value
Coefficient of variation (CV%) in neutral control wells: *(nmad/nmed)*100*	Inf%
POSITIVE CONTROL (well type = "p")	
Positive control well median response value, by plate: *pmed*	-100
Positive control well median absolute deviation, by plate: *pmad*	1.896
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: $((pmed - nmed) / sqrt(pmad^2 + nmad^2)$	-11.153
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: 845.

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:** Judson R, Houck K, Paul Friedman K, Brown J, Browne P, Johnston PA, Close DA, Mansouri K, Kleinstreuer N. Selecting a minimal set of androgen receptor assays for screening chemicals. Regul Toxicol Pharmacol. 2020 Nov;117:104764. doi: 10.1016/j.yrtph.2020.104764. Epub 2020 Aug 14. PMID: 32798611; PMCID: PMC8356084., Kleinstreuer NC, Ceger P, Watt ED, Martin M, Houck K, Browne P, Thomas RS, Casey WM, Dix DJ, Allen D, Sakamuru S, Xia M, Huang R, Judson R. Development and Validation of a Computational Model for Androgen Receptor Activity. Chem Res Toxicol. 2017 Apr 17;30(4):946-964. doi: 10.1021/acs.chemrestox.6b00347. Epub 2016 Dec 9. PMID: 27933809; PMCID: PMC5396026.

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.