ATG_zfER2b_XSP1

1. General Information

1.1 Assay Title: Attagene TRANS-FACTORIAL Multi-Species (XSP1) HepG2 Assay for zebrafish estrogen receptor (ESR2B)

1.2 Assay Summary: ATG_XSP1_multi-species_TRANS is a cell-based, multiplexed assay created by modifying the existing Attagene TRANS-FACTORIAL system to include a panel of nuclear receptors from some or all the following species: human (Homo sapiens), mouse (Mus musculus), frog (Xenopus laevis), zebrafish (Danio rerio), chicken (Gallus gallus), and turtle (Chrysemys picta). The ECOTOX-FACTORIAL format uses HepG2, a human liver cell line, with measurements taken at 24 hours after chemical dosing in a 24-well plate. Xsp1 included 3 nM 6-alpha-fluorotestosterone to partially stimulate the androgen receptor. ATG_zfER2b_XSP1 is one of 29 assay components measured or calculated from the ATG_XSP1_multi-species_TRANS assay. It is designed to make measurements of mRNA induction, a form of inducible reporter, as detected with fluorescence intensity signals by Reverse transcription polymerase chain reaction (RT-PCR) and Capillary electrophoresis technology. The assay endpoint ATG_zfER2b_XSP1 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, measures of mRNA for gain-of-signal activity can be used to understand the reporter gene at the transcription factor-level as they relate to the gene ER. Furthermore, this assay endpoint can be referred to as a primary readout, because this assay has produced multiple assay endpoints where this one serves a reporter gene function. 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: Attagene Inc. is a Contract Research Organization (CRO) offering a unique screening service using its proprietary multiplexed pathway profiling platform, the FACTORIAL.

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: FACTORIAL is a novel pathway profiling technology trademarked and patented by Attagene, Inc.

1.9 Assay Throughput: 24-well plate. Transfected HepG2 cells are aliquoted into 24-well microtiter plates and incubated with test compounds for 24 hours prior to PCR detection of total RNA transcription using capillary electrophoresis.

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 to fluorescence intensity signals are indicative of inducible changes in transcription factor activity. This is quantified by the level of mRNA reporter sequence unique to the trans-acting reporter gene response element ER, which is responsive to the zebrafish estrogen receptor. [GeneSymbol:esr2b | GeneID: 317733| Uniprot_SwissProt_Accession:Q5PR29].

Cellular adaptive response to environmental triggers is often mediated by an intracellular network of regulatory pathways that modulate gene expression. The signaling pathways interact with DNA by using transcription factors (TFs), or proteins that bind specific sequences on target genes. Assessing transcription factor activity can help characterize the functional status and impact of chemical exposures for expression of genes of interest.

2.2 Scientific Principles: Trans-FACTORIAL is an embodiment of the FACTORIAL platform that is designed for assessing agonist/antagonist properties of compounds across multiple NRs. The trans- FACTORIAL comprises a library of one-hybrid reporter constructs (trans-RTUs). A trans-RTU expresses a chimera GAL4-NR protein that regulates transcription of a reporter sequence. The presence of agonists/antagonists of NR alters the transactivation function of Gal4-NR and modulates reporter transcription.

2.3 Experimental System: adherent HepG2 cell line used. Hep G2 is an immortal cell line which was derived in 1975 from the liver tissue of a 15-year-old Caucasian male from Argentina with a well-differentiated hepatocellular carcinoma. These cells are epithelial in morphology, have a modal chromosome number of 55, and are not tumorigenic in nude mice. This cell line has been cloned and transfected with a library of multiple reporter transcription units.

2.4 Metabolic Competence: The HepG2 cells used in this assay are variant HG19, a cell line selected for enhanced xenobiotic metabolism. These cells express 2 to 13 times more cytochrome P450 activity than parental HepG2. The parental HepG2 cell line has been shown by others to retain the potential for Phase I and Phase II metabolic responses to xenobiotics, e.g., expression of CYP1A1/2, 2A6, 2B6, 2C8/9, 2C19, 2D6/3A, 2E1, and 3A4/5 with CYP1A2, CYP2C9, CYP2D6, CYP2E1 and CYP3A activities reported at levels similar to human hepatocytes although variable depending on source and culture conditions; some enzymes (e.g., CYP2W1) have even been observed at higher rates than in primary hepatocytes. Phase II enzyme activities identified in HepG2 cells include SULTS (1A1, 1A2, 1E1 and 2A1), GSTs (mGST-1, GST u1), NAT1, EPHX1 and UGTs (1A1, 1A6 and 2B7). In addition, HepG2 cells can potentially express xenobiotic regulation activities via functionally active p53 protein (Boehme et al. 2010) and Nrf2, a transcription factor which regulates genes containing antioxidant response element (ARE) sequences in their promoters; HepG2 cells also possess the capacity to express a number of ATP-binding cassette (ABC) xenobiotic export pumps (e.g., ABCC1, C2, C3 and G2 membrane-bound proteins also regulated in part by Nrf2 TF DNA-binding).

2.5 Exposure Regime: Human liver HepG2 cells are transiently transfected with multiple reporter transcription units (MRTUs) in 6-well plates using FuGene 6 reagent (Roche; 3 ml FuGene/1 mg DNA). The MRTU constructs are regulated by a cis-regulating element (promoter). Each RTU expresses a GAL4-UAS that regulates the transcription of a nearby target reporter gene sequence. A major difference between the CIS and TRANS system is that in CIS activities of endogenous TFs are measured, whereas the TRANS assay evaluates changes in activities of exogenous, chimeric NR-Gal4 proteins. Since the HepG2 cell line does not express some nuclear receptors, the CIS assay cannot be used to evaluate these targets. The transfected cells are pooled, plated onto a 96-well plates, and exposed to evaluated compound. At the end of 24 hour incubation, total RNA was isolated using TriZol reagent (Invitrogen). The isolated RNA is then reverse-transcribed using oligo(dT) primer and Mo-MLV reverse transcriptase (Invitrogen) with DNAse I (Ambion) treatment for 30 min. The one-tenth of the produced cDNA was amplified by PCR using Taq DNA polymerase (Invitrogen) and two reporter sequence-specific primers. The PCR products were fluorescently labeled by primer extension with 6-arboxyfluorescein (6-FAM) 5'-labeled reporter sequence-specific primer (2 min at 95 C, 20 s at 68 C and 10 min at 72 C) and these products were digested with 5U of HpaI (New England Biolabs) for 2h at 37 C. The fragments were purified using Qiaquick PCR columns (Qiagen), analyzed on an ABI 3130xL genetic analyzer (Applied Biosystems) with peak positions identified by using a set of X-rhodamine (ROX)-labeled MapMarker1000 molecular weight standards (BioVentures). The raw capillary electrophoresis data was processed using Attagraph software (Attagene).

ASSAY DESIGN SUMMARY	
Nominal number of tested concentrations:	Target (nominal) number of replicates:
8	1
Standard minimum concentration tested:	Standard maximum concentration tested:

0.835 µM	203 µM
Key positive control:	Neutral vehicle control:
NA	DMSO
Baseline median absolute deviation for the assay (bmad): 0.181	
Response cutoff threshold used to determine hit calls: 0.544	
Detection technology used: RT-PCR and Capillary electrophoresis (Fluorescence)	

2.6 Response: Increased transcription activity is measured by increased fluorescent intensity, specifically the increased production of mRNA transcripts production in response to active transcription following transcription factor (TF) interaction with promoter sequences as measured by reverse transcription-polymerase chain reaction (RT-PCR) and capillary electrophoretic detection of fluorescently labeled mRNA.

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:

Non-mammalian Vertebrate: Assays associated with non-mammalian vertebrate species

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: Readout data was analyzed in the positive (gain of signal) fitting direction using log2 fold-induction over DMSO controls which provide a baseline signal. Negative and zero values are removed before analysis and raw values are log transformed. All statistical analyses were conducted using R programming language, employing tcpl package to generate model parameters and confidence intervals. Each chemical concentration series was fit and the model which produces the lowest Akaike Information Criterion (AIC) value is considered the winning model.

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:

2: log2 (Transform the corrected response value (cval) to log-scale (base 2).), 3: rmneg (Exclude wells with negative corrected response values (cval) and downgrading their well quality (wllq); if cval < 0, wllq = 0.), 4: rmzero (Exclude wells with corrected response values (cval) equal to zero and downgrading their well quality (wllq); if cval = 0, wllq = 0.)

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:

1: bmad3 (Add a cutoff value of 3 multiplied by the baseline median absolute deviation (bmad) as defined at Level 4.), 3: log2_1.2 (Add a cutoff value of log2(1.2). Typically for fold change data.), 28: ow_bidirectional_gain (Multiply winning model hitcall (hitc) by -1 for models fit in the negative analysis direction. Typically used for endpoints where only positive 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: 100	Number of chemicals tested: 98

ACTIVITY HIT CALLS		
Active hit count: hitc≥0.9	Inactive hit count: 0≤hitc<0.9	NA hit count: *hitc<0*
82	14	*4*

WINING MODEL SELECTION	
Number of sample-assay endpoints with winning *hill* model:	25
gain-loss (gnls) model:	8
power(pow) model:	6
linear-polynomial (poly1) model:	11
quadratic-polynomial(poly2) model:	14
exponential-2 (exp2) model:	3
exponential-3 (exp3) model:	1
exponential-4 (exp4) model:	10
exponential-5 (exp5) model:	22

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*	0.912
Neutral control median absolute deviation, by plate: *nmad*	0.15
Coefficient of variation (CV%) in neutral control wells: *(nmad/nmed)*100*	16.41%
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: 22.

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:** Houck KA, Simha A, Bone A, Doering JA, Vliet SMF, LaLone C, Medvedev A, Makarov S. Evaluation of a multiplexed, multispecies nuclear receptor assay for chemical hazard assessment. Toxicol In Vitro. 2021 Apr;72:105016. doi: 10.1016/j.tiv.2020.105016. Epub 2020 Oct 10. PMID: 33049310., Medvedev AV, Medvedeva LA, Martsen E, Moeser M, Gorman KL, Lin B, Blackwell B, Villeneuve DL, Houck KA, Crofton KM, Makarov SS. Harmonized Cross-Species Assessment of Endocrine and Metabolic Disruptors by Ecotox FACTORIAL Assay. Environ Sci Technol. 2020 Oct 6;54(19):12142-12153. doi: 10.1021/acs.est.0c03375. Epub 2020 Sep 23. PMID: 32901485.

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.