Organisation for Economic Co-operation and Development

ENV/JM/MONO(2019)28

Unclassified **English - Or. English**

6 September 2019

ENVIRONMENT DIRECTORATE
JOINT MEETING OF THE CHEMICALS COMMITTEE AND THE WORKING PARTY ON CHEMICALS, PESTICIDES AND BIOTECHNOLOGY

Cancels & replaces the same document of 29 August 2019

CASE STUDY ON THE USE OF AN INTEGRATED APPROACH TO TESTING AND ASSESSMENT FOR ESTROGEN RECEPTOR ACTIVE CHEMICALS

Series on Testing and Assessment
No. 309

The corresponding annexes are available under the following cotes: ENV/JM/MONO(2019)28/ANN1

JT03450660

This document, as well as any data and map included herein, are without prejudice to the status of or sovereignty over any territory, to the delimitation of international frontiers and boundaries and to the name of any territory, city or area.

OECD Environment, Health and Safety Publications

Series on Testing and Assessment

No. 309

CASE STUDY ON THE USE OF AN INTEGRATED APPROACH TO TESTING AND ASSESSMENT FOR ESTROGEN RECEPTOR ACTIVE CHEMICALS

IOMC

INTER-ORGANIZATION PROGRAMME FOR THE SOUND MANAGEMENT OF CHEMICALS

A cooperative agreement among **FAO, ILO, UNDP, UNEP, UNIDO, UNITAR, WHO, World Bank and OECD**

Environment Directorate

ORGANISATION FOR ECONOMIC CO-OPERATION AND DEVELOPMENT

Paris 2019

About the OECD

The Organisation for Economic Co-operation and Development (OECD) is an intergovernmental organisation in which representatives of 36 industrialised countries in North and South America, Europe and the Asia and Pacific region, as well as the European Commission, meet to co-ordinate and harmonise policies, discuss issues of mutual concern, and work together to respond to international problems. Most of the OECD's work is carried out by more than 200 specialised committees and working groups composed of member country delegates. Observers from several countries with special status at the OECD, and from interested international organisations, attend many of the OECD's workshops and other meetings. Committees and working groups are served by the OECD Secretariat, located in Paris, France, which is organised into directorates and divisions.

The Environment, Health and Safety Division publishes free-of-charge documents in twelve different series: **Testing and Assessment; Good Laboratory Practice and Compliance Monitoring; Pesticides; Biocides; Risk Management; Harmonisation of Regulatory Oversight in Biotechnology; Safety of Novel Foods and Feeds; Chemical Accidents; Pollutant Release and Transfer Registers; Emission Scenario Documents; Safety of Manufactured Nanomaterials;** and **Adverse Outcome Pathways**. More information about the Environment, Health and Safety Programme and EHS publications is available on the OECD's World Wide Web site (www.oecd.org/chemicalsafety/).

This publication was developed in the IOMC context. The contents do not necessarily reflect the views or stated policies of individual IOMC Participating Organisations.

The Inter-Organisation Programme for the Sound Management of Chemicals (IOMC) was established in 1995 following recommendations made by the 1992 UN Conference on Environment and Development to strengthen co-operation and increase international co-ordination in the field of chemical safety. The Participating Organisations are FAO, ILO, UNDP, UNEP, UNIDO, UNITAR, WHO, World Bank and OECD. The purpose of the IOMC is to promote co-ordination of the policies and activities pursued by the Participating Organisations, jointly or separately, to achieve the sound management of chemicals in relation to human health and the environment.

This publication is available electronically, at no charge.

Also published in the Series on testing and Assessment link

For this and many other Environment,
Health and Safety publications, consult the OECD's
World Wide Web site www.oecd.org/chemicalsafety/

or contact:

OECD Environment Directorate,
Environment, Health and Safety Division
2, rue André-Pascal
75775 Paris cedex 16
France
Fax : (33-1) 44 30 61 80
E-mail : ehscont@oecd.org

© OECD 2019

Applications for permission to reproduce or translate all or part of this material should be made to: Head of Publications Service, RIGHTS@oecd.org, OECD, 2 rue André-Pascal, 75775 Paris Cedex 16, France

Forward

OECD member countries have been making efforts to expand the use of alternative methods in assessing chemicals. The OECD has been developing guidance documents and tools for the use of alternative methods such as (Q)SAR, chemical categories and Adverse Outcome Pathways (AOPs) as a part of Integrated Approaches for Testing and Assessment (IATA). There is a need for the investigation of the practical applicability of these methods/tools for different aspects of regulatory decision-making, and to build upon case studies and assessment experience across jurisdictions.

The objective of the IATA Case Studies Project is to increase experience with the use of IATA by developing case studies, which constitute examples of predictions that are fit for regulatory use. The aim is to create common understanding of using novel methodologies and the generation of considerations/guidance stemming from these case studies.

This case study was developed by the United States for illustrating practical use of IATA and submitted to the 2018 review cycle of the IATA Case Studies Project. This case study was reviewed by the project team. The document was endorsed at the 3rd meeting of the Working Party on Hazard Assessment in June 2019.

The following case study was also reviewed in the project in 2018:

1. CASE STUDY ON THE USE OF INTEGRATED APPROACHES TO TESTING AND ASSESSMENT FOR TESTICULAR TOXICITY OF ETHYLENE GLYCOL METHYL ETHER (EGME)-RELATED CHEMICALS, ENV/JM/MONO(2019)27, Series on Testing & Assessment No. 308

These case studies are illustrative examples, and their publication as OECD monographs does not translate into direct acceptance of the methodologies for regulatory purposes across OECD countries. In addition, these cases studies should not be interpreted as official regulatory decisions made by the authoring member countries.

In addition, a considerations document summarising the learnings and lessons of the review experience of the case studies is published with the case studies:

REPORT ON CONSIDERATIONS FROM CASE STUDIES ON INTEGRATED APPROACHES FOR TESTING AND ASSESSMENT (IATA) -Fourth Review Cycle (2018) -, ENV/JM/MONO(2019)26, Series on Testing & Assessment No. 307.

This document is published under the responsibility of the Joint Meeting of the Chemicals Committee and Working Party on Chemicals, Pesticides and Biotechnology.

Executive Summary

Endocrine disrupting chemicals are a diverse set of substances that have the potential to interfere with normal endocrine function and lead to an adverse outcome. Due to the potential impact of these adverse effects, regulatory agencies in many countries evaluate endocrine activity of environmental chemicals for specific regulatory endpoints. As part of the Integrated Approach to Testing and Assessment (IATA) project, this document presents a case study to evaluate potential estrogenic activity from environmental chemicals. The IATA presented in this document has characteristics that are consistent with a defined approach (DA) and describes an integrated testing strategy (ITS) for the identification of endocrine disruption by a substance primarily for the purposes of screening and prioritisation without the use of animal testing. The data interpretation procedure (DIP) is designed to provide information on whether the substance tested may act as an agonist of estrogen receptor activation. The combination of test methods used covers multiple key events (KEs) of the pathway leading to ER agonist activity. The prediction model combines at least four results obtained from *in vitro* assays addressing different KEs of the pathway to determine the final classification. Computational model classifications were derived for 1812 substances, and model performance was compared to high quality *in vitro* and *in vivo* data for specific reference chemicals. Depending on the combination of tests used, this prediction model generally achieved accuracies of 84 to 93%, as compared to reference chemical data from US EPA's Endocrine Disruptor Screening Program (EDSP) Tier 1 guideline *in vitro* tests and uterotrophic assay. These results compellingly verify the applicability of this testing strategy as an IATA for identification of estrogenic chemicals.

Table of contents

Forward 6

Executive Summary 7

Acronym List 10

Introduction 11

1. Purpose 13

1.1. Purpose of Use 13
1.2. Target Chemical(s) 13
1.3. Endpoint(s) 13

2. Hypothesis for Performing the IATA 14

3. Approaches Used 15

4. Data/Information Gathering 17

5. Application of IATA 25

5.1. Summary of Data 25
5.2. Application of IATA 32
5.3. Strengths and Limitations of this IATA 33
5.4. Uncertainty 34
5.5. Strategy and Integrated Conclusion 36

6. Acknowledgements 38

7. References 39

Annex I. Detailed assay descriptions in OECD GD211 Format 42

Annex II. Curated reference chemical lists. 43

Annex III. The Possible Combinations of 7 or Fewer Assays that Can Be Used in this IATA 45

Annex IV. Main Scientific Papers 46

Tables

Table 1. ER pathway Model Proposed as Alternative to Specific OECD Test Guidelines 13
Table 2. Summary of the *in vitro* assays used with their ID mapping to the model in Figure 3. 19
Table 3. Minimum criteria for guideline-like (GL) uterotrophic studies (Kleinstreuer et al., 2016). ... 24
Table 4. Performance Based Validation of the ER pathway Model Based on 16 High-Throughput *in vitro* Assays Measuring Potential Estrogen Receptor (ER) Agonist Activities and *in vitro* Reference Chemicals [a] 28
Table 5. Uncertainty of Defined Approach 34
Table AII-1. *In vitro* Estrogen Receptor (ER) Agonist Reference Chemicals 43
Table AII-2. *In vivo* Estrogen Receptor (ER) Agonist Reference Chemicals with at Least two Independent Active or Inactive Guideline-like Uterotrophic Studies (Browne et al., 2015) 44

Figures

Figure 1. Representation of the ER pathway and computational model in the context of the molecular initiating event (MIE) and associated key events (adapted from Browne et al. 2017). 16
Figure 2. Conceptual diagram of the overall process for development, validation, and application of the IATA.......... 17
Figure 3. Schematic diagram of the integrated model of chemical perturbations in the estrogen receptor agonist pathway (Judson et al., 2015). 22
Figure 4A. Activity of the *in vitro* reference chemicals (Judson et al., 2015).......... 26
Figure 4B. Activity of *in vivo* reference chemicals (Browne et al., 2015). 27
Figure 5. Performance of the best computational model utilizing different numbers of *in vitro* HTS assays from 1 to 16 (Judson et al., 2017). 31
Figure 6. Flow chart for analysis using this IATA. 33
Figure 7. ER pathway model results for chemicals with an AUC (Agonist) value > 0.1 (Watt and Judson, 2018).......... 36

Acronym List

AIC	Akaike Information Criteria
AUC	Area Under the Curve
AOP	Adverse Outcome Pathway
ATG	Attagene
BA	Balanced Accuracy
CEPA	Canadian Environmental Protection Act, 1999
DA	Defined Approach
DIP	Data Integration Process
DMSO	Dimethyl Sulfoxide
EDSP	Endocrine Disruptor Screening Program
EPA	Environmental Protection Agency
ER	Estrogen Receptor
ERTA	Estrogen Receptor Transactivation
EU	European Union
FFDCA	Federal Food, Drug and Cosmetic Act (US)
FIFRA	Federal Insecticide, Fungicide and Rodenticide Act (US)
GL	Guideline-Like
HTS	High-throughput Screening
IATA	Integrated Approach to Testing and Assessment
ICCVAM	Interagency Coordinating Committee on the Validation of Alternative Test Methods
ITS	Integrated Test Strategy
KE	Key Event
MAD	Mutual Acceptance of Data
MIE	Molecular Initiating Event
NCATS	National Center for Advancing Translational Sciences
NVS	Novascreen
OECD	Organisation for Economic Cooperation and Development
OT	Odyssey Thera
OVX	Ovariectomised
PBTG	Performance-Based Test Guideline
PND	Postnatal Day
REACH	Registration, Evaluation, Authorisation and Restriction of Chemicals (EU)
TG	Test Guideline
US	United States

Introduction

Endocrine disrupting chemicals are a diverse set of substances that have the potential to interfere with normal endocrine function, which may lead to adverse outcomes (e.g., impaired reproduction). The endocrine system is highly integrative and complex, with variability in activity following exposure, depending on the organ or timepoint studied. Due to the potential impact of these adverse outcomes, health effects following exposure to endocrine-active substances have been the subject of intensive study and have been described in numerous research papers and reviews (e.g., Colborn and Clement 1992; Kavlock et al., 1996; WHO 2002; WHO/UNEP 2012; Hotchkiss et al., 2008; Soto and Sonnenschein 2010; Gore et al., 2015a). Beyond these and other research publications, there are also numerous guidance documents and standardised test guidelines published on the evaluation of endocrine disrupting chemicals.

The Organisation for Economic Co-operation and Development (OECD) has investigated test methods in the scientific literature that could be standardised and used in chemical regulations to detect and characterise hazards posed by endocrine disrupting chemicals. A Conceptual Framework for Testing and Assessment of Endocrine Disrupters (as revised in 2012) lists the OECD Test Guidelines (OECD 2012a) and standardised test methods available, under development or proposed to be used to evaluate chemicals for endocrine disruption. These tests include both *in vitro* and *in vivo* assays that examine the mechanisms, pathways and adverse effects on endocrine relevant endpoints. The majority of these tests focus on a limited number of endocrine pathways at this time, including estrogen, androgen, thyroid and steroidogenesis. Included in these guidance documents are OECD technical guideline documents for assays that measure the effect of the tested substance on upstream and downstream steps associated with estrogen receptor activation, including receptor binding and transactivation, and organ effects (e.g., uterine weight changes).

The ability to quickly assess chemicals for endocrine disruption is a global need. Under the European Union's (EU) Registration, Evaluation, Authorisation and Restriction of Chemicals (REACH) regulation, endocrine disruptors are considered to be substances of high concern. Similar importance is given to endocrine disruptors by Canada, the United States (US) and many other countries. For example, the European Commission has recently finalised a communication on science-based criteria for endocrine disruptors based on recent regulations (EC 2018), and the EU has recently introduced specific legislative obligations aimed at phasing out endocrine disruptors in water, industrial chemicals, plant protection products and biocides. In Canada, the Canadian Environmental Protection Act, 1999 (CEPA) places mandatory obligations on the Minister of Health and the Minister of the Environment concerning research on hormone disrupting substances, and, when available, information on endocrine disrupting effects has been considered in the risk assessments of substances (CEPA 1999). Similarly, in the US, endocrine disrupting effects are an important area of research and considered in risk assessments of many substances. For example, US EPA developed the Endocrine Disruptor Screening Program (EDSP) in response to the statutory mandate in the Federal Food, Drug, and Cosmetic Act (FFDCA) to evaluate activity against endocrine toxicity pathways through a series of tiered *in vitro* and *in vivo* tests for specific regulatory endpoints (US EPA 2002). For these and other regulatory agencies, determination of endocrine disrupting effects from a specific chemical may lead to the restriction of its use.

Given the number of potential endocrine-disrupting substances in the environment that need to be analysed, there is a need to validate and incorporate more rapid and cost-effective methods to help accelerate the analysis of these substances. In order for new approach methodologies to be used in regulatory decision making, it is necessary to demonstrate that these methods are scientifically sound and can effectively detect endocrine activity, e.g., via nuclear hormone receptor-mediated interactions.

As part of the Integrated Approach to Testing and Assessment (IATA) project, this document presents a case study to evaluate potential estrogenic activity of environmental chemicals. The IATA presented in this document has characteristics that are consistent with a defined approach (DA) which do not rely on expert judgement, so yield reproducible results. This IATA describes an integrated testing strategy (ITS) for the identification of endocrine disruption by a substance primarily for the purposes of screening and prioritisation without the use of animal testing. The data interpretation procedure (DIP) is designed to provide information on whether the substance tested may act as an agonist of estrogen receptor activation. The combination of up to 16 *in vitro* high-throughput screening (HTS) assays covers multiple key events (KEs) of the pathway leading to estrogen receptor (ER) activation. The prediction model integrates results from at least four *in vitro* HTS assays addressing different KEs of the pathway to determine the final classification. The integration of at least four assays increases the accuracy of the prediction model and minimises the technical limitations of any one method. This case study describes the design, performance-based evaluation, and application of this IATA.

1. Purpose

1.1. Purpose of Use

The case study uses a combination of *in vitro* HTS assays and a computational model for ER activity, which could serve as an alternative to low and medium throughput *in vitro* and *in vivo* tests. The original model consisted of 16 *in vitro* HTS assays that encompassed multiple points on the ER pathway. Results from this model were analysed, and the performance validated against sets of *in vitro* and *in vivo* reference chemicals to determine if they provide an accurate quantitative measure of ER agonist activity for the chemicals assayed. The goal is for the model to serve as an alternative to the current guideline ER binding (TG493; OECD, 2015b), ER transactivation (ERTA; TG455; OECD, 2015a) and uterotrophic assays (TG440; OECD, 2007) (Table 1). Further evaluation of the model performance demonstrated that this could be achieved with subsets of *in vitro* HTS assays as long as the HTS assays used in the subset fit specific criteria (discussed in more detail below). The intended application of this IATA is screening and priority setting of environmental chemicals based on their ER agonist activity and for further evaluation of endocrine-related activity in higher tier *in vivo* tests (e.g., female pubertal assay, two generation reproductive toxicity study).

Table 1. ER pathway Model Proposed as Alternative to Specific OECD Test Guidelines

PBTG493	ER Binding	*Performance-Based Test Guideline for Human Recombinant Estrogen Receptor (hrER) In vitro Assays to Detect Chemicals with ER Binding Affinity*	OECD, 2015b
PBTG455	ER Transactivation	*Performance-Based Test Guideline for Stably Transfected Transactivation In vitro Assays to Detect Estrogen Receptor Agonists and Antagonists*	OECD, 2015a
TG440	Uterotrophic assay	*Uterotrophic Bioassay in Rodents: A Short-Term Screening Test for Oestrogenic Properties*	OECD, 2007

1.2. Target Chemical(s)

The target chemicals are environmental and commercial chemicals.

1.3. Endpoint(s)

The endpoint being assessed is ER agonist activity following exposure to a specific chemical as measured in a subset of *in vitro* HTS assays run in US EPA's ToxCast program. The original model used a suite of 16 HTS assays that measure the molecular initiating event (i.e., receptor binding), in addition to several key events (e.g., receptor dimerisation, DNA binding, transactivation, gene expression, and cell proliferation) in the ER pathway (Figure 1). Further analysis determined that the use of models integrating multiple combinations of 4 to 16 HTS assays can accurately predict the estrogenic activation potential of a chemical. The criteria for selection of the HTS assays used is that they probe diverse points in the ER pathway, and use diverse assay measurement technologies and cell types.

2. Hypothesis for Performing the IATA

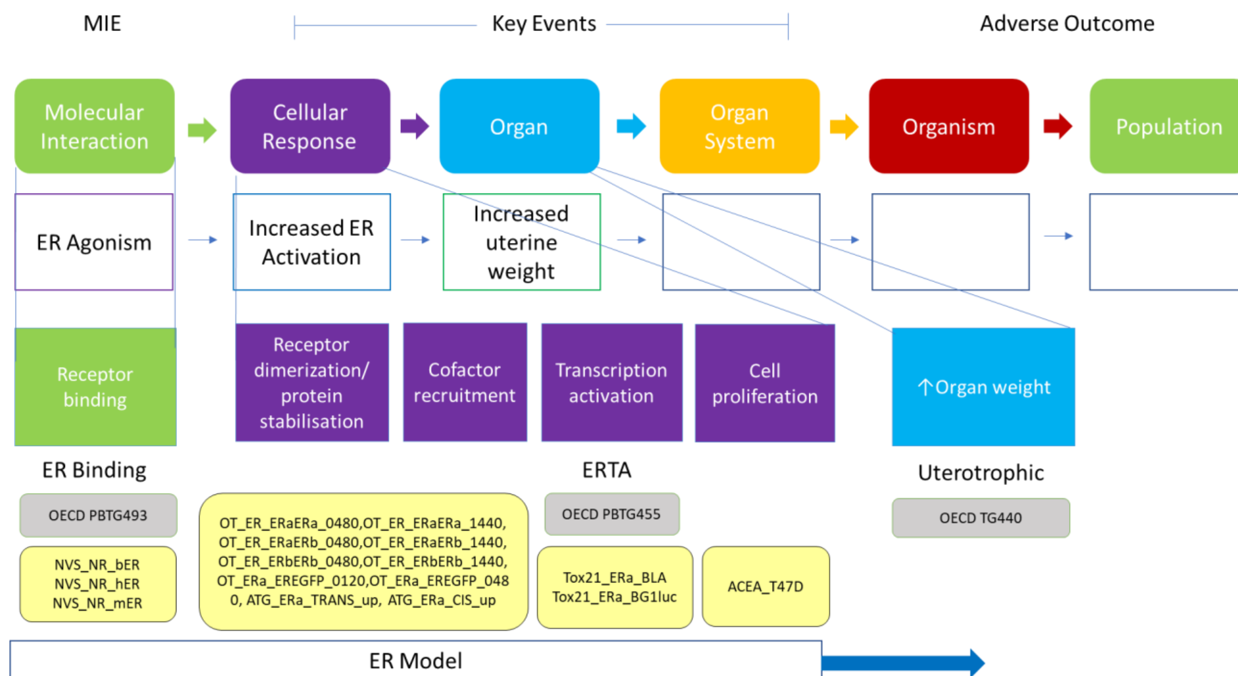
Adverse toxicological outcomes are driven by interactions between chemicals and biomolecular targets, which can be better understood through the use of AOPs. Using an AOP as a framework, chemical perturbations of key events may be estimated or measured by *in silico* models, biochemical assays, cell-based *in vitro* assays, and *in vivo* animal models. In the case of the ER pathway model contained in this case study, *in vitro* HTS assays are designed to characterise the impact of environmental chemicals on the molecular initiating events or early key events in the ER pathway (Figure 1). In order to reduce the potential of false positives, the assays probe multiple steps along the pathway, often with replicate coverage of each key event. This case study is based on a series of HTS assays integrated into a computational model of cellular KE in the upstream events of the ER pathway. Therefore, the hypothesis for performing this IATA was to use a pathway-based, computational model (based on *in vitro* HTS assays) to be able to screen and prioritise chemicals for further assessment, based on whether or not they demonstrated ER agonist activity. This methodology allows for a more rapid analysis of a larger number of chemicals than was possible with traditional, low-throughput *in vitro* or *in vivo* assays. Consequently, more resources can be focused on further analysis of those chemicals that show evidence of potential endocrine disruption.

3. Approaches Used

ER Pathway

The use of a pathway approach for designing high throughput *in vitro* assays and new computational tools is a valuable way to determine potential endocrine disruption effects following exposure to environmental chemicals. Multiple adverse outcome pathways (AOPs) are under development related to ER bioactivity (http://aopwiki.org). This IATA was developed based on the known biology of the estrogen receptor signalling pathway including reproductive, developmental and other health effects. The health effects following exposure to endocrine-active substances have been well-studied and are described in numerous research papers and reviews (e.g., Colborn and Clement 1992; Kavlock et al., 1996; WHO 2002; WHO/UNEP 2012; Hotchkiss et al., 2008; Soto and Sonnenschein 2010; Gore et al., 2015a). For this case study, we evaluated ER agonist activity using data from a collection of 16 *in vitro* assays that probe the ER pathway downstream from ER binding in mammalian systems. The computational model used 16 *in vitro* assays which are a subset of a larger collection of assays used in the US EPA ToxCast program ((821 individual assay endpoints; Dix et al., 2007; Judson et al., 2010; Kavlock et al., 2012). Figure 1 depicts the ER pathway and key events that were the basis of the computational model, as well as the mapping of the *in vitro* HTS assays to the respective key events.

Figure 1. Representation of the ER pathway and computational model in the context of the molecular initiating event (MIE) and associated key events (adapted from Browne et al. 2017).



ER pathway showing MIE and key events and how they relate to OECD technical guidance (grey squares). Perturbation of the MIE and downstream key events (KEs) are evaluated using high-throughput screening (HTS) assays (yellow squares). ER = Estrogen receptor; ERTA = ER transactivation; MIE = molecular initiating event.

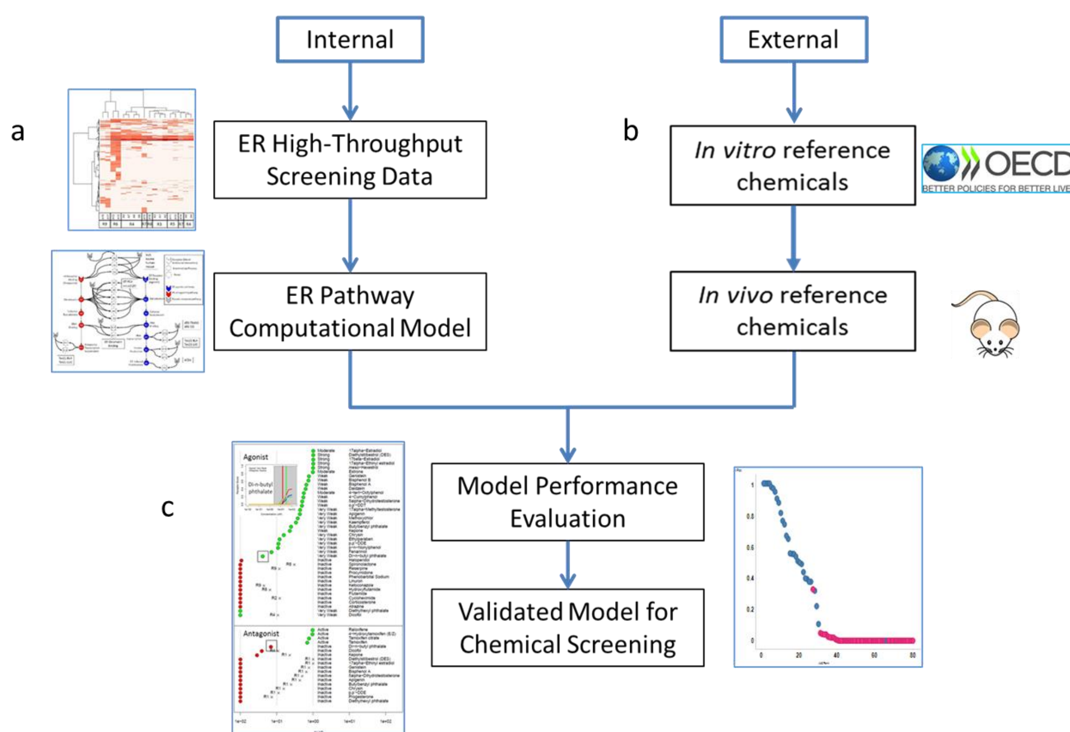
Integrated Approach to Testing and Assessment (IATA)

The IATA presented in this document has characteristics consistent with a DA and describes an ITS for the identification of potential endocrine disruption through ER activation by a substance. The use case for the IATA is for the purpose of screening and prioritisation without the use of animal testing. The DIP is currently designed to provide information on whether the substance tested may act as an estrogen receptor agonist. The DIP uses data from the individual *in vitro* HTS assays (ranging from 4 to 16 assays), which are then integrated in the computational model (as described in detail in Judson et al., 2015; 2017). The score from the computational model is used to identify and quantify the ER agonist activity of a chemical. The combination of test methods employed in the IATA covers the MIE and multiple KEs in the pathway, and can be used as alternatives to 3 existing guideline studies (including 1 *in vivo* TG). For a positive result, the computational model requires concordant results for at least 4 assays in separate KEs (or MIE). The performance of the *in vitro* / computational model of 1812 substances was evaluated by comparison to high quality *in vitro* and *in vivo* data for specific reference chemicals (Kleinstreuer et al., 2016, Browne et al., 2015, Judson et al., 2015; 2017). More details of this IATA are described below.

4. Data/Information Gathering

The IATA described here is an integrated battery of *in vitro* HTS assays and a computational model of ER pathway activation. The case study evaluates the development, validation, and application of the integrated battery of HTS assays and computational model for determining potential endocrine disruption. The overall approach is shown in Figure 2, with details on the specific steps included below. The data, model and validation performance are all peer-reviewed, published, and transparent, with all the underlying data and code made publicly accessible. The more detailed publications are cited where appropriate.

Figure 2. Conceptual diagram of the overall process for development, validation, and application of the IATA.



a. HTS assays were developed to encompass multiple points on the ER pathway and were used to assay the ToxCast chemical set (1800+). The results of these assays are integrated through the ER pathway model to yield a prediction value of ER agonist activity of a chemical.
b. *In vitro* and *in vivo* reference chemicals were curated for use in a performance-based evaluation of the model. Full description of the curation of these lists is described in the text.
c. A performance-based evaluation of the model was then conducted using the reference chemicals and EDSP Tier 1 chemicals in the ToxCast chemical set.

In vitro Assays. Details of the *in vitro* assays are described on US EPA's ToxCast Web site (US EPA, 2015a) and in a variety of publications (Dix et al., 2007; Judson et al., 2010). Detailed descriptions of these assays, including their biological targets and any proprietary aspects, can be found in Annex I. Briefly, potential ER activation was measured in 16 high-throughput *in vitro* assays run in US EPA's ToxCast program. The suite of high throughput assays measures the MIE (i.e., receptor binding), in addition to several KEs (e.g., receptor

dimerisation, DNA binding, transactivation, gene expression, and cell proliferation) in the ER pathway (Figure 1). The 16 ER assays include three cell-free biochemical radioligand ER binding assays; three protein complementation assays that measure formation of ER dimers and test for activity against ERα and ERβ, each measured at two time points; an assay measuring interaction of the mature transcription factor with DNA at two time points; two reporter gene assays measuring RNA transcript levels; two assays measuring reporter protein levels; and an ER-sensitive cell proliferation assay (Table 2; assay IDs correspond to Figure 3). The assay sources refer to the company or laboratory where the assays were performed. All *in vitro* and *in vivo* reference chemicals were run in the 16 high-throughput ER assays. In addition to the *in vitro* and *in vivo* reference chemicals, a total of 1812 chemicals were also run across the full set of 16 ER assays. All chemicals were run in a blinded fashion in all the assays.

This set of biochemical and cell-based *in vitro* assays relies on different technologies and probes different key events in the ER signalling pathway. Although the assays primarily use human proteins and/or cell types, the suite of 16 assays include human, murine, and bovine ER binding assays and ER pathway interactions in a variety of human tissue types (Table 2). Every assay is potentially subject to technology-specific interference (e.g., chemicals that denature the receptor protein, are luminescent, are cytotoxic, etc.) that can be mistakenly interpreted as ER-specific activation. Conceptually, assay interference (Auld et al., 2008; Baell and Holloway, 2010; Bruns and Watson, 2012; Thorne et al., 2010) is a phenomenon whereby assays designed to measure binding to a protein or perturbation of a given pathway may produce false signals when the target protein itself, or other pathways in the system, are altered non-specifically. For instance, a chemical could cause protein denaturation, which could give rise to a false positive signal in cell-free, radioligand competitive-binding assays. Such a chemical would show activity in all assays of that technology, but not the cell-based assays. Another example would be fluorescent compounds, which would show false activity in all fluorescence-based assays. The standard approach to deal with assay interference issues is to deploy “orthogonal” assays (Miller et al., 2010; Thorne et al., 2010) that help distinguish activity towards the intended target or pathway from non-specific activities. In addition to assay interference issues, every assay has inherent limitations such as dynamic range or levels of background noise. Using a suite of assays to detect pathway perturbations may minimise potential non-specific effects or limitations of any single assay (Judson et al., 2015).

Table 2. Summary of the *in vitro* assays used with their ID mapping to the model in Figure 3.

Further details are provided in Annex I.

Assay ID	Assay Name	Source	Gene Target	Normalised Data Type	Assay Design Type	Biological Process Target	Detection Technology	Detection Technology Subtype	Measurement Timepoint (Hours)	Organism	Tissue	Cell Format	Cell Line Name	Assay Footprint
A1	NVS_NR_bER	NVS	ESR1	percent activity	radioligand binding	receptor binding	Lysate-based radiodetection	Scintillation counting	18	bovine	uterus	Tissue based cell-free	NA	microplate: 96-well plate
A2	NVS_NR_hER	NVS	ESR1	percent activity	radioligand binding	receptor binding	Lysate-based radiodetection	Scintillation counting	18	human	NA	cell-free	NA	microplate: 96-well plate
A3	NVS_NR_mERa	NVS	Esr1	percent activity	radioligand binding	receptor binding	Filter-based radiodetection	Scintillation counting	18	mouse	NA	cell-free	NA	microplate: 96-well plate
A4	OT_ER_ERaERa_0480	OT	ESR1	percent activity	protein fragment complement-ation assay	protein stabilisation	Protein-fragment Complementation	Fluorescence intensity	8	human	kidney	cell-free	HEK293T	microplate: 384-well plate
A5	OT_ER_ERaERa_1440	OT	ESR1	percent activity	protein fragment complement-ation assay	protein stabilisation	Protein-fragment Complementation	Fluorescence intensity	24	human	kidney	cell line	HEK293T	microplate: 384-well plate
A6	OT_ER_ERaERb_0480	OT	ESR1	percent activity	protein fragment complement-ation assay	protein stabilisation	Protein-fragment Complementation	Fluorescence intensity	8	human	kidney	cell line	HEK293T	microplate: 384-well plate
A7	OT_ER_ERaERb_1440	OT	ESR1 ESR2	percent activity	protein fragment complement-ation assay	protein stabilisation	Protein-fragment Complementation	Fluorescence intensity	24	human	kidney	cell line	HEK293T	microplate: 384-well plate
A8	OT_ER_ERbERb_0480	OT	ESR2	percent activity	protein fragment complement-ation assay	protein stabilisation	Protein-fragment Complementation	Fluorescence intensity	8	human	kidney	cell line	HEK293T	microplate: 384-well plate

A9	OT_ER_ERbERb_1440	OT	ESR2	percent activity	protein fragment complement-ation assay	protein stabilisation	Protein-fragment Complementation	Fluorescence intensity	24	human	kidney	cell line	HEK293T	microplate: 384-well plate
A10	OT_ERa_EREGFP_0120	OT	ESR1	percent activity	fluorescent protein induction	regulation of gene expression	Microscopy	Optical microscopy: Fluorescence microscopy	2	human	cervix	cell line	HeLa	microplate: 384-well plate
A11	OT_ERa_EREGFP_0480	OT	ESR1	percent activity	fluorescent protein induction	regulation of gene expression	Microscopy	Optical microscopy: Fluorescence microscopy	8	human	cervix	cell line	HeLa	microplate: 384-well plate
A12	ATG_ERa_TRANS_up	ATG	ESR1	log2 fold induction	mRNA induction	regulation of transcription factor activity	RT-PCR and Capillary electrophoresis	Fluorescence intensity	24	human	liver	cell line	HepG2	microplate: 24-well plate
A13	ATG_ERE_CIS_up	ATG	ESR1	log2 fold induction	mRNA induction	regulation of transcription factor activity	RT-PCR and Capillary electrophoresis	Fluorescence intensity	24	human	liver	cell line	HepG2	microplate: 24-well plate
A14	Tox21_ERa_BLA_Agonist_ratio	Tox21	ESR1	percent activity	beta lactamase induction	regulation of gene expression	GAL4 b-lactamase reporter gene	Fluorescence intensity	18	human	kidney	cell line	HEK293T	microplate: 1536-well plate
A15	Tox21_ERa_LUC_BG1_Agonist	Tox21	ESR1	percent activity	luciferase induction	regulation of gene expression	Luciferase-coupled ATP quantitation	Bioluminescence	22-24	human	ovary	cell line	BG1	microplate: 1536-well plate
A16	ACEA_T47D_80hr_Positive	ACEA	ESR1	percent activity	real-time cell-growth kinetics	cell proliferation	RT-CES	Electrical Sensor: Impedance	80	human	breast	cell line	T47D	microplate: 96-well plate

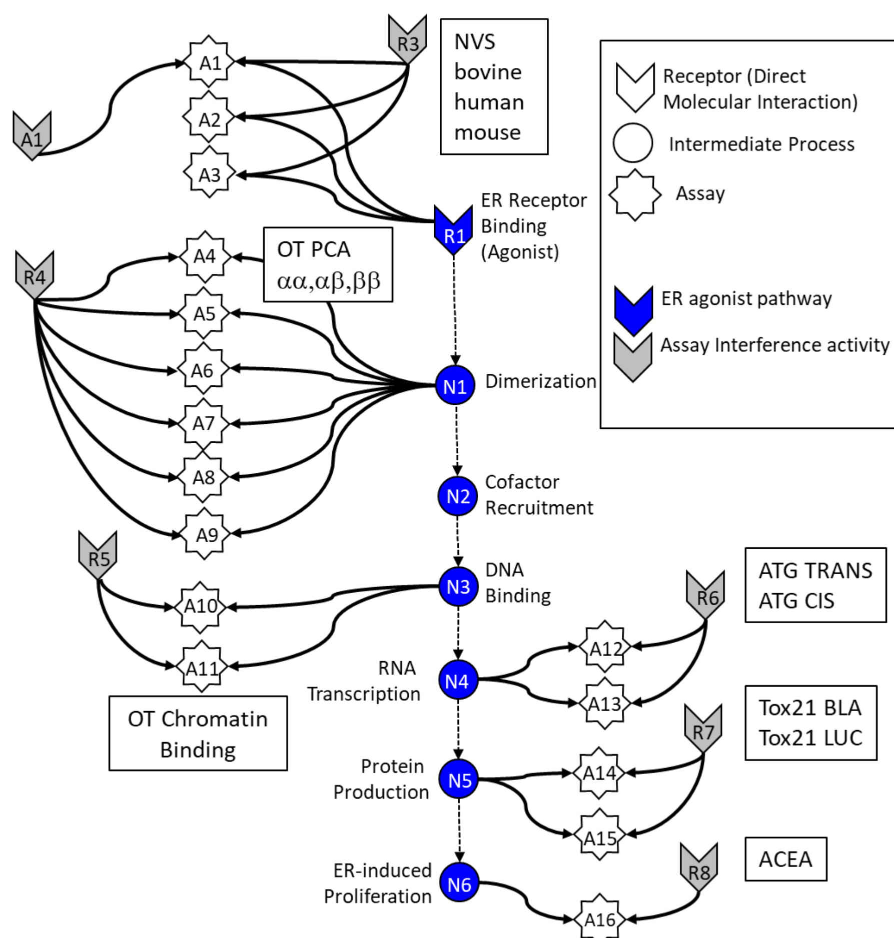
NVS=Novascreen; OT=Odyssey Thera; ATG=Attagene; Tox21=assays run by the National Institutes of Health's National Center for Advancing Translational Sciences (NCATS) as part of the Federal Tox21 program.

Chemicals were run in concentration−response format in all assays except the cell-free binding assays. Cell-free competitive binding assays were initially run at a single screening concentration (25 µM), and if the test chemical was active in the assay (i.e., radioligand was displaced), the assay was run in concentration−response format. All *in vitro* assays except the assays measuring RNA transcript levels were normalised to 17β-estradiol. RNA transcript data were normalised as a fold-change from the solvent (DMSO) control. Concentration−response data from *in vitro* assays were fit to three statistical models that included a four parameter Hill model, a modified Hill model with gain-loss at high concentrations, or a constant (no concentration−response) model. The best model was statistically selected using the Akaike Information Criteria (AIC) value. All concentration−response data were analysed using the ToxCast data analysis pipeline (tcpl; Filer 2017), which automates the processes of baseline correction, normalisation, curve-fitting, hit-calling, and detection of a variety of potential confounders. The data analysis pipeline is available for download (https://doi.org/10.23645/epacomptox.6062788). To integrate results of the different *in vitro* assays, synthetic concentration−response curves were generated for each assay across 14 standardised concentrations from 0.01 to 100 µM.

Computational Modelling. The concentration−response curves for all 16 assays were included in a computational model, referred to here as the ER pathway model. The model code is available for download (https://doi.org/10.23645/epacomptox.6062542). The model itself is conceptually simple, and its workings can be described with the aid of Figure 3 (Judson et al., 2015). We assume that a chemical can act in several ways: it can be a true ER agonist, a true ER antagonist (not described here), it can cause false activity in one or more assays through some assay interference mechanism, or it can be totally inactive. There are three kinds of nodes in Figure 3. The blue nodes are the series of known biological processes in the ER agonist pathway, from Figure 1. The grey nodes (assay interference activity in Figure 3) are technology specific assay interference processes that a chemical could trigger (technology being a combination of cell type and assay readout). The white, star-shaped nodes are the assays. Lines connecting ER or interference nodes with assay nodes indicate which assays will be activated if one of the biological nodes (agonist or interference) is activated. For instance, if a chemical is a true agonist, all assays (A1-A16) should be active, because arrows connect the ER receptor binding / agonist node with all of the assay nodes. However, if a chemical is not an agonist, but instead interferes with the technology of assays A1-A3 (all cell-free radioligand binding assays) through interference process R3, then assays A1-A3 will be activated, but none of the other assays will be. The mathematical model that combines all of the assay data takes the pattern of activity seen across the assays and determines the most likely cause (agonist, antagonist, interference through one of the other nodes). This process is carried out is using a straightforward mathematical optimisation function, and is done for each concentration separately. This step then yields a concentration-response curve for each of the agonist, antagonist and interference nodes for each chemical. The area under the curve (AUC) for a chemical-node pairs is then just the integral of the corresponding concentration-response curve. This mathematical process does not require that all assays connected to a node be active, but the weight assigned to the node will increase with the fraction of corresponding assays that are turned on. An important detail of the model is that it picks the smallest set of modes that explain the data. The AUC values are normalised so that the ER agonist AUC "model scores" range from 0 (no activity) to 1 (activity of 17β-estradiol). Model scores were truncated at values <0.001, considered to have no ER activation and given a score of 0. A model score of <0.001 implies a concentration required to elicit 50% of the maximal response (AC50) greater than 10 mM which is several orders of magnitude greater than the highest concentrations tested in the ER assays. ER pathway agonist AUC scores ≥0.1 were

considered positive. A model score of 0.1 equates to an AC50 of ~100 µM, which is the upper limit of testing in the assays. Model scores of 0.1 > AUC > 0.001 were considered inconclusive because these chemicals were active in only one or two ER assays and activity was limited to the highest concentrations tested. Activity in only a few of the 16 assays may be due to differential sensitivities in the various *in vitro* assays, although all chemicals were tested up to at least 100 µM. The model was applied to the ToxCast library of 1812 chemicals, including reference chemicals described below, and correctly identified the agonists except for weak agonists whose activity was outside the concentration range tested (Judson et al., 2015).

Figure 3. Schematic diagram of the integrated model of chemical perturbations in the estrogen receptor agonist pathway (Judson et al., 2015).



The input data for the model includes concentration-response data for 16 ER-related *in vitro* assays. The model produces scores for agonist activity (blue) and for assay interference activity (grey). These scores can then be compared with reference chemicals, structure classes, and other properties.

Curation of lists of reference chemicals for in vitro and in vivo ER agonist activity (Judson et al., 2015; Browne et al., 2015; Kleinstreuer et al., 2016): ER pathway model performance was evaluated for both *in vitro* and *in vivo* reference chemicals, as well as results of EDSP Tier 1 screening assays in current practice, in order to evaluate the ability of the ER pathway model to accurately identify active and inactive chemicals based on previous *in vitro*, *in vivo* and EDSP Tier 1 screening. The set of 40 *in vitro* reference

chemicals were previously identified using multiple validated low throughput *in vitro* ER assays, and were identified by the Interagency Coordinating Committee on the Validation of Alternative Test Methods (ICCVAM) and OECD for the express purpose of validating novel *in vitro* assays. Forty chemicals (28 agonists of differing potencies indicated by a range in AC50 values (activity concentration required to elicit 50% maximal response) and 12 inactive chemicals) were selected for reproducible results in *in vitro* ER binding and transactivation assays, and to include a diverse set of chemical structures. The set of *in vitro* reference chemicals includes the list used in the OECD TG457 BG1 guidance document (OECD 2012b). The complete set of *in vitro* reference chemicals used in this study are given in Annex II (Browne et al. 2015).

The set of 43 *in vivo* reference chemicals (30 positive, 13 inactive) were identified from current (up to Dec 2014) scientific literature search for studies that measured uterine weight changes in immature or ovariectomised (OVX) rats, or OVX mice (See list in Annex II; Browne et al., 2015). Literature searches were performed on over 1800 chemicals (using the ToxCast Phase I/Phase II/E1K chemical library as starting point). This resulted in over 700 papers, which were further evaluated for specifically defined quality control metrics for inclusion in a final database of "guideline-like" (GL) studies. GL studies were those that were compliant with the uterotrophic study design from the OECD TG 440 (OECD 2007), and adhered to six pre-defined minimum criteria (i.e., animal model used, route of administration, necropsy timing, dosing interval, number of dose groups, and group size; Table 3). These are further defined in Kleinstreuer et al. (2016). Studies that met all 6 minimum criteria were considered and compiled into single database (Kleinstreuer et al., 2016). Study quality evaluation was based on information extracted from each publication, and independently scored by two scientists. It should be noted that compliance with the minimum criteria identified is not necessarily equivalent to a thorough assessment of overall study quality, as it did not consider internal validity of each study, risk of bias or difference in biotransformation due to route of administration.

As part of this analysis, both the qualitative and quantitative reproducibility of a uterotrophic assay across many chemicals tested at many different laboratories was assessed. Of the 70 chemicals in the database with at least two reported GL uterotrophic studies, 18 (26%) had at least one study with a discordant outcome, resulting in a chemical being classified as both "active" and "inactive" for uterotrophic bioactivity. Discordant outcomes could result from differences in overall study protocol design and/or from the range of doses tested in each study. This analysis provides a good example of the high degree of variability that can be seen in the uterotrophic bioassay (Kleinstreuer et al., 2016), and provides a basis for comparison when evaluating alternative approaches as a replacement for the animal test.

Table 3. Minimum criteria for guideline-like (GL) uterotrophic studies (Kleinstreuer et al., 2016).

Animal Models	Route of Administration	Necropsy Timing	Dosing Interval	Number of Dose Groups	Group Size
OVX Adult Rat: OVX 6-8 weeks, 14-day post-surgery recovery *OVX Adult Mouse*: OVX 6-8 weeks, 7-day post-surgery recovery *Immature Rat*: Begin dosing postnatal day 18-21, complete dosing by PND 25	Oral gavage Subcutaneous injection Intraperitoneal injection	Between 18-36 hours after last dose	Dosing for minimum of three consecutive days; must be completed by PND 25 in immature animals	Minimum of two dose groups, must have positive and negative control groups	*Control groups*: minimum three animals *Treatment groups*: minimum five animals

Abbreviations: OVX, ovariectomised; PND, postnatal day.

5. Application of IATA

5.1. Summary of Data

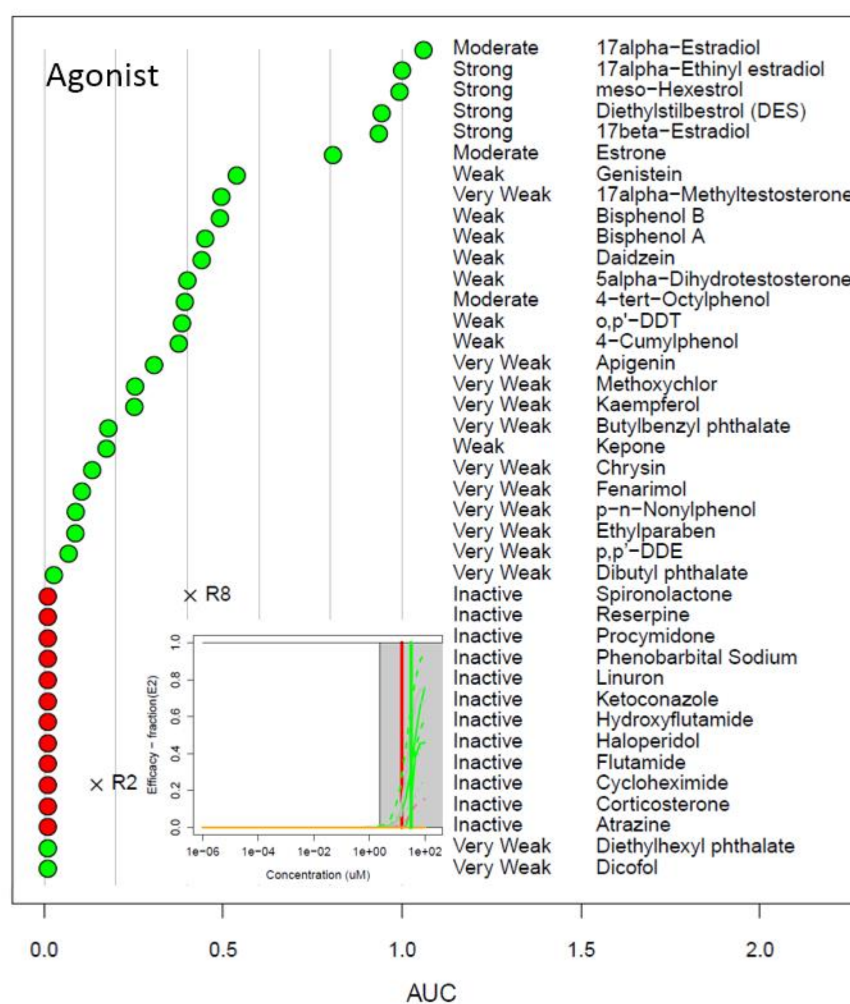
Performance-based evaluation and application of the ER pathway model. The ER pathway model is not developed by fitting to data from a set of training chemicals. Instead, the ER pathway model considers the expected patterns of assay activity for different modes (agonist, antagonist, several types of assay interference), based on an understanding of the underlying biology, as described above. The model then evaluates the pattern for each chemical and assigns the chemical to the mode with the highest score. Reference chemicals are used to validate this scoring approach and not to train the model. This IATA case study provides evidence to validate this IATA, with most of the validation work performed using all 16 assays. After validation of the 16-assay ER pathway model, research was conducted to determine the minimal number of assays for acceptable predictivity. This work demonstrated that as few as 4 assays are sufficient by comparing the results from various combinations of 1 to 15 assays to results from the 16-assay ER pathway model and US Tier 1 screening assays (Judson et al., 2017).

The performance of this ER pathway model was evaluated using a validation approach consistent with the OECD conceptual framework for assessing potential endocrine disrupting chemicals (OECD 2012a). The ER pathway model results were compared to three sets of reference chemicals: (1) a set of reference chemicals with consistent ER agonist activity from multiple reports *in vitro* ER assays in the literature (*in vitro* reference chemicals) (Annex II; Browne et al., 2015; Judson et al., 2015; 2017); (2) a set of reference chemicals with *in vivo* guideline uterotrophic data from multiple labs from the literature (*in vivo* reference chemicals) (Annex II; Browne et al., 2015; Kleinstreuer et al., 2016; Judson et al., 2017), and (3) a set of chemicals with results of data from US EDSP Tier 1 guideline ER assays (i.e., ER binding assay, ER transcriptional activation assay, ER uterotrophic assay; Browne et al., 2015). Reliability of the model results were measured for each evaluation. This analysis focused on the ER agonist activity from the model, and included analysis of true positives, true negatives, false positives, false negatives, balanced accuracy (average of sensitivity and specificity), sensitivity and specificity. This analysis focused on those chemicals with an indication of ER agonist activity (AUC > 0.1) or no activity (AUC = 0) but excluded inconclusive results (0.001 < AUC < 0.1). ER pathway model agonist scores were examined for 1812 chemicals evaluated in the 16 ER assays. The 1812 chemicals also include pesticide active ingredients (387) and pesticide inerts (367). The results of this evaluation are described below.

In vitro reference chemicals from the literature (Judson et al., 2015). As part of the performance evaluation of the ER pathway model, the model results for ER agonists were compared to a set of 40 positive and negative reference chemicals that were assayed for ER agonist activity (i.e., ER binding assay, ER transcriptional activation assay). The AUC values for these reference chemicals are plotted in Figure 4A. For the 40 *in vitro* reference chemicals, the ER pathway model performed with an overall balanced accuracy of 93%. Of the 28 active reference chemicals, 26 of 28 showed ER agonist activity with agonist AUC>0.1. As above, the ER agonist scores were positive (≥0.1) for all strong, moderate and weak agonist reference chemicals. Two very weak reference chemicals (diethylhexyl phthalate, dicofol) were false negatives, and one very weak active chemical (di-butyl phthalate) was inconclusive (0.001 < AUC < 0.1). Of the 12 inactive reference chemicals,

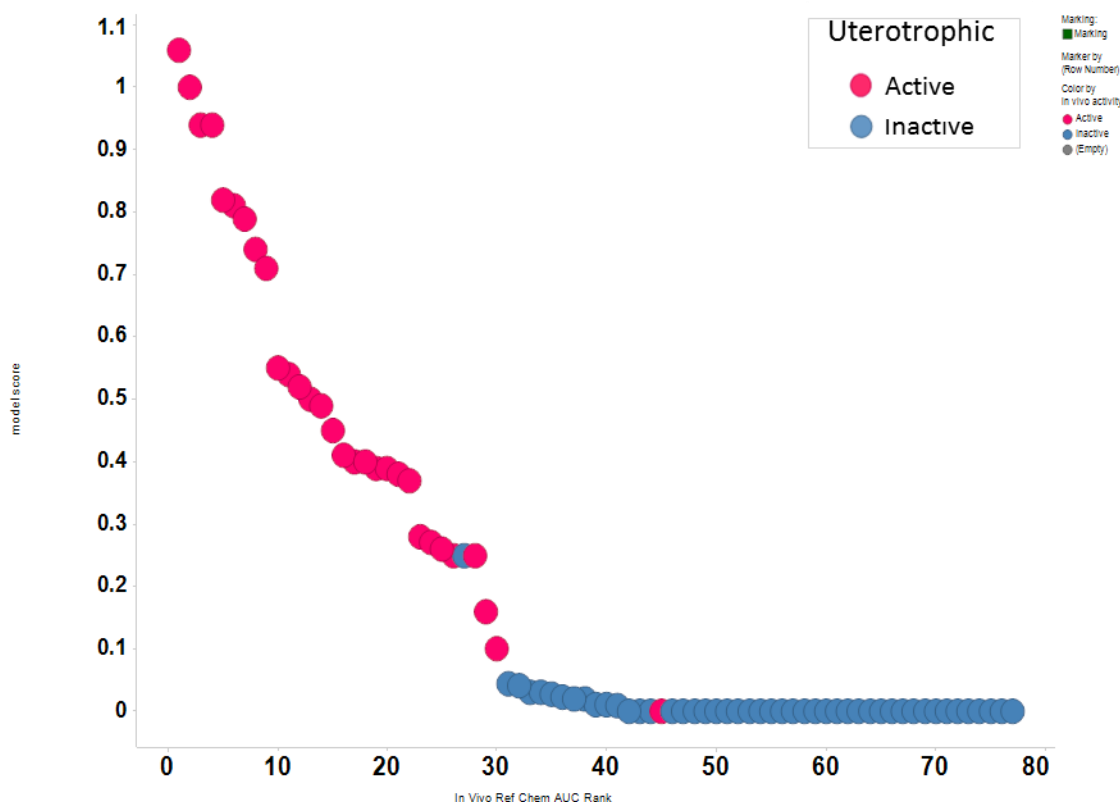
11 chemicals had ER agonist scores of zero. One inactive chemical (haloperidol) had an inconclusive pathway model score (0.001 < AUC < 0.1). If the two chemicals with inconclusive ER agonist model scores are excluded from performance metrics, then the overall balanced accuracy is 95%.

Figure 4A. Activity of the *in vitro* reference chemicals (Judson et al., 2015).



Positive reference chemicals are indicated by green circles, while negative reference chemicals are indicated by red circles. For the agonists, the expected potency range is also indicated (middle column). For chemicals with one or more assay interference AUC values greater than zero, the value is indicated by an X, and the assay interference node is indicated.

Figure 4B. Activity of *in vivo* reference chemicals (Browne et al., 2015).



Chemicals are plotted according to their ER model score (ER agonist AUC) and color-coded based on their uterotrophic response (active = pink, inactive = blue).

Guideline uterotrophic assay reference chemicals (Browne et al., 2015; Kleinstreuer et al., 2016). To further evaluate the ER pathway model, the model agonist scores were compared with 103 chemicals run in at least one guideline-like uterotrophic study (Figure 4B). This larger set of chemicals included 43 *in vivo* reference chemicals with independently verified results in two or more guideline-like uterotrophic studies. The overall accuracy of the ER pathway model agonist scores evaluated for these 43 chemicals was 86%. Of 30 active reference chemicals, 29 had positive ER pathway model scores (AUC ≥ 0.1). The potential false negative chemical, octamethylcyclotetrasiloxane (D4), was positive for uterine weight gain in multiple uterotrophic studies run in independent laboratories but negative in the ER pathway model. It is possible that the volatility of this chemical reduced the concentration of this compound actually tested in the high throughput assay. One possibly false positive, kaempferol, was negative for uterine weight gain in uterotrophic studies but had a modest ER pathway model agonist score (AUC = 0.25). This positive result was consistent with other lower throughput *in vitro* ER assays of this chemical, and may be indicative of a false negative response in the animal data due to the tissue-specific nature of kaempferol activity (i.e., it does not act on uterine tissue but does have an effect on others ER-responsive tissues). Four inactive *in vivo* reference chemicals (dibutyl phthalate, dicyclohexyl phthalate, dihexyl phthalate, and fenvalerate) had very low ER pathway model scores associated with inconclusive calls (0.001 < AUC < 0.1). This result supports the hypothesis that ER pathway model in this range has limited *in vivo* relevance. If the four inconclusive chemicals were excluded from calculations of performance metrics, then the resulting

overall accuracy for model performance for *in vivo* reference chemicals was 95% (37/39). To further examine the accuracy of the ER pathway model agonist scores against *in vivo* assays, the model results were also compared with a further 60 chemicals run in only one guideline-like uterotrophic study. With this analysis, the overall accuracy of the ER pathway model was 84% (86/103).

US EDSP Tier 1 reference chemicals (Browne et al., 2015). Within the ToxCast chemicals analysed by the ER pathway model, there were 52 from the EDSP Tier 1 list that have data from the EDSP Tier ER binding, ERTA, and uterotrophic assays. Comparing ER pathway model scores and EDSP Tier 1 results, three List 1 chemicals did not have ToxCast assay data and none of the remaining 49 chemicals had ER pathway model scores ≥0.1. Similarly, none of the chemicals had clear positive agonist activity in the Tier 1 ER *in vitro* assays (ER binding and ERTA) or *in vivo* (uterotrophic) assays (Table 4). ER pathway model scores were inconclusive for eight List 1 Tier 1 chemicals, all of which had limited signal in the EDSP Tier 1 assays, but none of which would be considered positive based on the Tier 1 response. All ER assay responses for these chemicals were detected at concentrations similar to those that resulted in cytotoxicity and may be explained by cell-stress or cytotoxicity-related false positive activity. Although there were both positive and negative Tier 1 ERTA assays reported for two chemicals, there were not clear indications of a positive Tier 1 ER binding, ERTA, or uterotrophic study (or any study submitted to US EPA to satisfy a Tier 1 test order) for any chemical. Similarly, ER pathway model scores were negative for the remaining 41 chemicals. Comparison between computational methods and Tier 1 assays is biased by the lack of positive results, but for this analysis the model accuracy against List 1 chemicals with Tier 1 data is 84% (41/49) and 100% if inconclusive results are not included.

Table 4. Performance Based Validation of the ER pathway Model Based on 16 High-Throughput *in vitro* Assays Measuring Potential Estrogen Receptor (ER) Agonist Activities and *in vitro* Reference Chemicals [a]

performance	*in vitro* reference chemicals	*in vivo* reference chemicals	GL uterotrophic studies	Tier 1 studies
# true pos	26 (25)	29 (29)	49 (38)	0 (0)
# true neg	11 (11)	8 (8)	37 (37)	41 (41)
# false pos	1 (0)	5 (1)	11 (4)	8 (0)
# false neg	2 (2)	1 (1)	6 (6)	0 (0)
accuracy	0.93 (0.95)	0.86 (0.95)	0.84 (0.88)	0.84 (1.0)
sensitivity	0.93 (0.93)	0.97 (0.97)	0.89 (0.86)	0 (0)
specificity	0.92 (1.0)	0.67 (0.89)	0.77 (0.90)	0.84 (1.0)

a: ER pathway model scores ≥0.1 were considered positive, negative scores = 0 (and values <0.001 were truncated as 0), and model scores (0 > AUC < 0.1) were inconclusive. Performance metrics were calculated with all chemicals and excluding chemicals with inconclusive model scores (values shown in parentheses).

In sum, the full ER pathway model accuracy was 84% to 93% concordant when compared to reference chemicals and predicted results of EDSP Tier 1 guideline and other uterotrophic studies with a minimum of 84% up to 100% accuracy if inconclusive chemicals are excluded (Table 4). The performance of high-throughput assays and ER pathway model predictions demonstrates that these methods correctly identify active and inactive reference chemicals, provide a measure of relative ER agonist activity, and rapidly identify chemicals with potential endocrine bioactivities for additional screening and testing. When applied to a larger set of environmental/commercial chemicals, ~7% have

ER pathway model score indicative of agonist activity (Data not shown; Browne et al., 2015 Supplemental).

Reduced subset of HTS assays. The ER pathway model was also examined to determine whether a subset of assays could achieve equivalent performance to the full 16 *in vitro* HTS assays used in the agonist-mode. A simple additive model of ER agonist activity using variable numbers of assays was developed and calibrated against the full ER pathway model. The subset model produces a chemical-specific ER agonist AUC value using the formula:

$$AUC(chemical) = \sum_{i=0}^{n} a_i \times log_{10}(AC_{50}^{i}(chemical)) \times T^{i}(chemical)$$

where AC_{50}^{i} is the potency of the chemical and T^{i} is the corresponding "top" parameter or efficacy in assay *i*. The weight parameters a_i are constants for the specific subset model, and n is the number of assays in that subset model, and are provided in the supplemental material of Judson et al. (2017).

Separate "subset models" for all combinations of 1, 2, … 16 assays (65,535 in total) were then evaluated against the full model, using balanced accuracy as the performance metric (active vs. inactive). The results of this analysis demonstrated that there were many subsets of assays that performed at levels comparable to the full 16-assay model. The simplest, high-performing subsets used just 4 assays. As long as the subset of assays probe diverse points in the ER pathway and use diverse assay reporting technologies and cell types, there is flexibility in constructing this integrated battery of *in vitro* assays that can still yield equivalent accuracy in predicting estrogen agonist activity. Performance was evaluated for sensitivity, specificity and balanced accuracy of the subset model relative to the specific classifications of the full model, the *in vitro* reference chemicals and the *in vivo* reference chemicals described above. This is described in more detail in Judson et al. (2017).

In general, analysis of all models showed that it was possible to achieve 96% balanced accuracy (all chemicals vs full model) for certain combinations of 7 or more *in vitro* assays. Performance with some models with 4, 5 and 6 assays was almost as high. Analysis of just the *in vitro* reference chemicals vs the full model is better on average, and demonstrated that there are sets of as few as 4 assays that correctly classify all of the *in vitro* reference chemicals. Further analysis of the subset models showed that those that are optimised for one metric (e.g., classification of the reference chemicals vs full model), may have suboptimal performance on other metrics, such as classification of non-reference chemicals.

Figure 5 depicts the performance metrics for the best model for each number of assays from 1 to 16. The 'best' model shown is the subset model with the highest minimum balanced accuracy (i.e., minimum value for the BA for *in vitro* literature-based reference chemicals, *in vivo* literature-based reference chemicals, and all reference chemicals versus the full model). The results of this analysis demonstrated that there were many subsets of assays that performed at levels comparable to the full 16-assay model, including some that included as few as 4 assays. Annex III contains a table with all subset models with 7 or fewer assays that achieve BA ≥94% for the full chemical set and for the *in vitro* and *in vivo* subsets. Almost all of these models use the cell-free human and bovine radioligand binding assays, the ATG TRANS transactivation assay and the ACEA cell proliferation assay. None of the subset models use the Tox21 transactivation assays. The main difference between these models is which of the Odyssey Thera dimerisation assays were used. It is important to note that none of the models gives exactly the same classification as the full model. While almost all of the false negative reference chemicals in both the full model and the

subset models are due to the limited upper testing concentration (~100µm), potential causes of a subset false positive call may vary. A subset false positive chemical is one where the subset model indicates the chemical is positive for estrogenicity, while the full model indicates that it is negative. The fewer assays used in a model, the more weight one false positive assay may carry overall as it will not be compensated for by a large number of presumably negative assays. False positives may be those that are selective estrogen receptor modulators but functionally classified as estrogen antagonists (e.g., tamoxifen citrate), or chemicals that are active in assays sensitive to these chemical classes in the absence of estrogen receptor activity (e.g., progesterone or glucocorticoid-activating chemicals). Selective estrogen receptor modulators have high full-model antagonist AUC scores (Judson et al., 2015), and are expected to have significant activity in the upstream assays in the pathway (binding, protein dimerisation, chromatin binding), but limited to no activity in the downstream agonist assays (transactivation, proliferation) (Judson et al., 2015). However, progesterone or glucocorticoid-activating chemicals are active mostly in the ACEA cell proliferation assay (Judson et al., 2017). This assay, run in T47D cells, is known to be sensitive to these classes of chemicals independent of the estrogen receptor. Other chemicals that are detergents or solvents may be activating the *in vitro* assays through a cell stress or technology interference pathways. Most of these "false positive" chemicals are principally active in the ATG assays, as observed previously (Judson et al., 2015). One hypothesis about these chemicals is that they are actually estrogenic, but only after bioactivation. The ATG assays are run in a sub-strain of HepG2 cells that was selected for increased CYP activity. More details on this analysis can be found in Judson et al. (2017).

The results of this analysis show that there are a variety of *in vitro* HTS assay subsets that can be used to accurately classify chemicals for ER agonist activity. It is acceptable for this IATA to use as few as 4 assays and concurrent cytotoxicity/ cellular proliferation assay. Several nodes of the AOP are being evaluated alongside a phenotypic response (cellular proliferation). Different signal detection systems and different platforms (cell types/cell free systems) are used in the analysis (see Annex III for acceptable combinations). As long as the subset of assays interrogate different points on the ER pathway and incorporate different technologies, there is flexibility in constructing this integrated battery of *in vitro* HTS assays that can still yield equivalent accuracy in predicting ER agonist activity. This flexibility of the model would allow users to incorporate assays that they currently use or easily have access to as long as the criteria described above is met. A further aspect of the model flexibility allows for a decrease in potential barriers from required use of *in vitro* HTS assays with proprietary information.

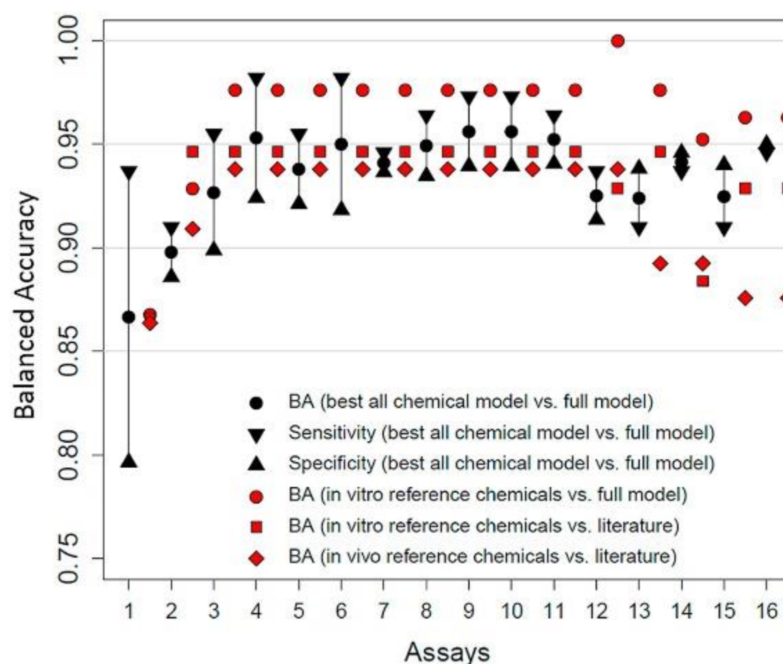


Figure 5. Performance of the best computational model utilizing different numbers of *in vitro* HTS assays from 1 to 16 (Judson et al., 2017).

The black symbols indicate data for the best all-chemical model. The red symbols are shifted to the right slightly for ease of visualisation. Figure shows the balanced accuracy for the best model using fewer than the 16 *in vitro* assays as compared to the full model for all ToxCast chemicals, and *in vitro* and *in vivo* reference chemicals. BA=balanced accuracy.

Applicability domain. In this context applicability domain describes the chemical space in which an assay correctly reflects the underlying molecular interactions of a chemical. Chemical classes that give false positive results in an assay technology would then be out of the applicability domain for that assay. An example would be fluorescent chemicals which can give false positive signals in fluorescence-based assays. The ER pathway model integrates several assays for the MIE and multiple KEs across an adverse outcome pathway to predict chemical toxicity for that pathway. Therefore, this model is less likely to result in false negatives or positives, as this integration takes into account the potential that an "inactive" chemical in one assay may have been "active" in another assay targeting a different point in the pathway. Conversely, a chemical that is positive in one assay through assay interference will be negative in the remaining assays that use different technologies. Therefore, while the applicability domain of a single high throughput assay may be limited, the computational model integrates multiple assays that encompass the broader toxicity pathway, and therefore can extend the applicability domain. However, the majority of the *in vitro* HTS assays used in the case study lack metabolic competence. Further, the assays used here are currently DMSO-based. Therefore, chemicals that require bioactivation for ER agonist activity may not be detected, and ER agonists that are not soluble in DMSO have not been tested.

Application of the IATA allows the rapid screening of many chemicals for estrogen bioactivity. Based on the performance-based validation of this model, multiple chemicals can be tested using the HT *in vitro* assays that encompass the ER pathway as described above, and the data integrated through the ER pathway model. The result of this IATA is a prediction of whether the chemical analysed has estrogen receptor agonistic bioactivity

along with a proposed potency. If yes, the recommendation to the regulator would be to prioritise for further analysis. If no, this would be screened as a lower priority for further testing. This information may be of use to many regulatory agencies to assist in identifying the specific endocrine mode of action when required by the regulatory decision context and to protect public health and the environment.

The regulatory relevance and application of this IATA is to serve as a tool for screening for priority setting in view of further evaluation, as well as to serve as an alternative test to the current guideline estrogen receptor (ER) binding (TG493; OECD, 2015b), ER transactivation (ERTA; TG455 OECD, 2015a) and uterotrophic assays (TG440; OECD, 2007). This is Tier 1 through 3 in OECD (for estrogen receptor agonistic bioactivity). In June 2015, the US EPA accepted the ER pathway model for screening of bioactivity, where it can substitute for the following Tier 1 assays: uterotrophic (OCSPP 890.1600), endocrine receptor binding (OCSPP 890.1250), and endocrine receptor transactivation assays (OCSPP 890.1300accepted that the ER pathway model gives similar predictive results for these tests (US EPA, 2015b). Recent research is ongoing to investigate the potential for using the full ER pathway model to predict ecological effects from ER agonist activity (Dreier et al., 2017). Research in this area may broaden applicability of the case study for predicting the impact of endocrine disruption of ecological endpoints like the reproductive outcomes in fish.

Suitability for use in regulatory decisions. The ER pathway model has already undergone extensive expert review. There have been several peer-reviewed scientific publications relating to the ER pathway model, as listed in the References. In January 2013 in the US, the initial work on this ER pathway model was presented to the FIFRA SAP. Many concerns of the SAP were addressed, and the ER pathway model was again presented to the FIFRA SAP in December 2014. In June 2015, the US EPA accepted the ER pathway model for screening of bioactivity, where it can substitute for the following Tier 1 assays: uterotrophic (OCSPP 890.1600), endocrine receptor binding (OCSPP 890.1250), and endocrine receptor transactivation assays (OCSPP 890.1300). The FR notice announcing the acceptance can be found here, and the 890-series guidelines can be found here. Further refinement of the ER pathway model has occurred since June 2015, and more research is being performed (such as adding metabolic competency to the assays).

In this case study, the performance of the ER pathway model has been evaluated against current low and medium throughput guideline assays and demonstrated to have similar accuracy for determining ER agonist activity of the tested substances. The use of this ER pathway model has already been implemented by the US EPA as alternatives to some EDSP Tier 1 testing (binding, ERTA, uterotrophic), and was included as a preferred data source in recent ECHA/EFSA guidance for estrogen-related endocrine activity (ECHA and EFSA 2018). The *in vitro* high throughput and computational model alternatives provide a focused evaluation of the mechanistic aspects of the ER pathway, thereby providing specific and quantitative measures of ER agonist activity. As other regulatory agencies are exploring the use of HTS assays and computational models in regulatory decisions, this ER pathway model may be used as an alternative for prioritisation in order to inform these decisions, and may also be used to provide information on the specific endocrine mode of action (i.e. estrogen receptor agonism) when required by the regulatory decision context.

5.2. Application of IATA

Application of the IATA allows the rapid screening of many chemicals for ER agonist activity (Figure 6). Based on the performance-based validation of this model, multiple

chemicals can be tested using as few as 4 HTS *in vitro* assays that encompass multiple KE in the ER pathway as described above, and the data integrated through the ER pathway model. The result of this IATA is a prediction of whether the chemical analysed shows ER agonist activity along with an estimated potency. This IATA may also be used as an alternative to existing OECD technical guidelines (e.g., TG 440). This information may be of use to multiple regulatory agencies in order to screen and prioritise substances for potential endocrine disruption.

Figure 6. Flow chart for analysis using this IATA.

The general steps necessary for using this IATA to determine ER agonist activity of selected chemicals. Following selection of *in vitro* HTS assays to be used, run these assays along with the cytotoxicity assays and then evaluate this data in the ER pathway model and generate a report consistent with those requested by regulatory agencies.

5.3. Strengths and Limitations of this IATA

The strengths and weaknesses of this IATA are described in more detail in the publications referenced above. The following are the main strengths and limitations:

Strengths:

- Non-animal integrated testing strategy.

- ER pathway model validated against a large number of reference chemicals.
- Lower quantities of test material needed for testing compared to animal tests.
- Pathway-based, more informative on mechanism of action.
- Predictivity of the ER pathway model ranges from 84 – 93% when compared to reference chemicals described above.

Limitations:

- Technical aspects of the assays (e.g., chemical compatibility with vehicle).
- Lack of metabolic competence in the assays.
- Focused on one endocrine disruption pathway (i.e., ER agonist activity).
- Simplified assays for inferring integrated physiological responses.
- Evaluation for non-receptor binding and events upstream of ER binding not incorporated.

5.4. Uncertainty

There are uncertainties associated with various aspects of this IATA, including the ER pathway, *in vitro* HTS assays, computational model and data analysis. The uncertainties, assumptions, and confidence in each of these aspects are described in Table 5.

Table 5. Uncertainty of Defined Approach

Factor	Uncertainty (low, medium, high)	Comment
Hypothesis used for the Defined Approach case study	Low	The hypothesis for performing this IATA was to use a pathway-based, computational model (based on *in vitro* HTS assays) to be able to screen and prioritise chemicals for further assessment, based on whether or not they demonstrated ER agonist activity. This methodology allows for a more rapid analysis of a larger number of chemicals than was possible with traditional, low-throughput *in vitro* or *in vivo* assays.
Assumptions related to key events of ER pathway	Low	Pathway frameworks are built upon assumptions of a relationship between the MIE, subsequent KEs and the proposed apical effect or adverse outcome. The use of these pathways may not consider that there are unknown pathways that may impact the decisions made about a specific substance. While it is important to consider the assumptions related to specific pathways during any analysis, pathways are useful as a framework for organizing the data to aid in highlighting where existing assays can be used to inform decisions, and where gaps may exist that require the development of new assays.
Reference chemicals for performance evaluation of the ER model	Low	Number of reference chemicals used for the performance evaluation of the ER model is sufficient. Well documented curation of reference chemicals based on their ER activity is included in the references cited in this case study. Study quality was evaluated but should be noted that this evaluation is not necessarily equivalent to a thorough assessment of overall study quality.
In vitro HTS assays and computational model	Low-medium	All testing methods have some element of uncertainty. In the analysis of the HTS assays, statistical methods are being developed to establish uncertainty bounds around potency and efficacy values. These statistical methods involve resampling the data and refitting the concentration response curves thousands of times to quantitatively estimate the uncertainty. Concentration-response parameters such as potency and efficacy are extracted from HTS data using nonlinear regression, and

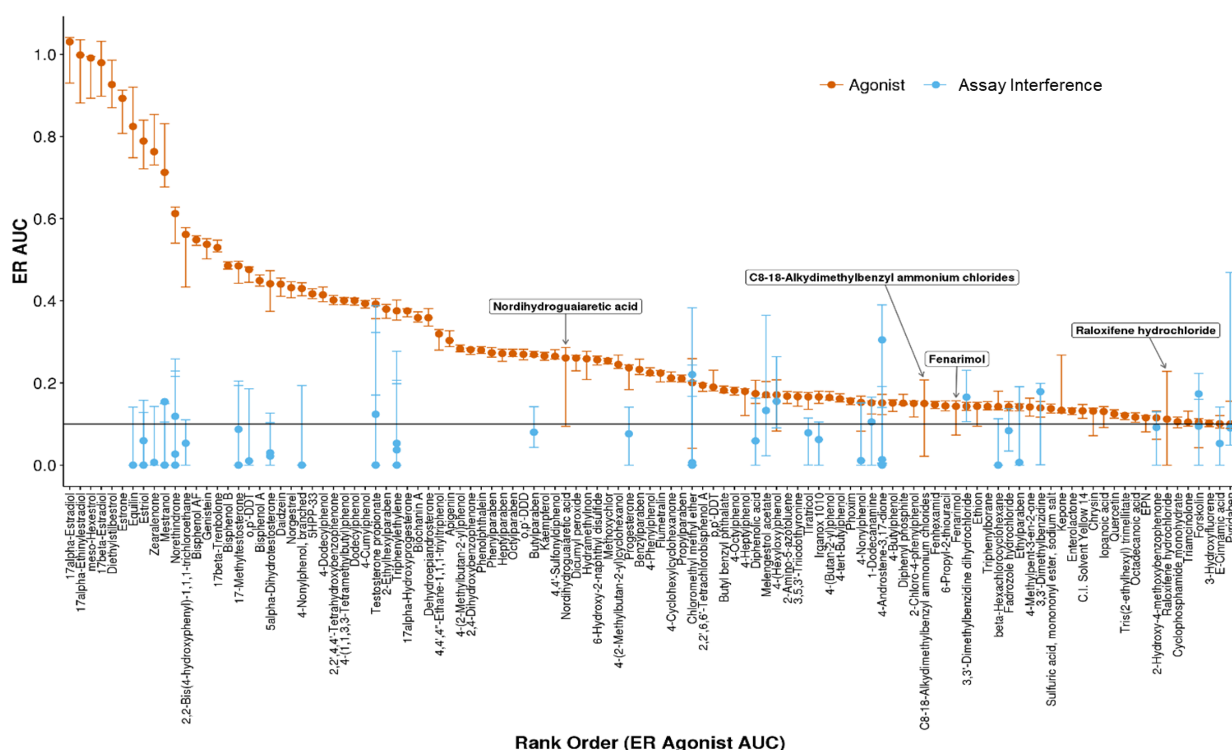
		models and analyses built from these parameters are used to predict *in vivo* and *in vitro* toxicity of thousands of chemicals. This is described in more detail in the text. HTS uncertainty is discussed in Watt and Judson (2018)
Technical Limitations of *in vitro* HTS assays	Low-medium	A common criticism of the *in vitro* HTS assays relates to technical limitations of the *in vitro* HTS assay format, including limitations on testing all chemicals due to solubility in test media, and the lack metabolic competence, which may lead to over- or underestimation of chemical hazard. There is ongoing research to address using these assays with water-soluble compounds. The latter is a common criticism of all *in vitro* assays, and one that is addressed in low-throughput assays with the addition of metabolic enzyme systems to the assays (both extracellularly and intracellularly). For the HTS assays, multiple approaches are also being evaluated to endow a range of cell types and cell lines with the required metabolic activity.
Concordance and weight of evidence of all data used for justifying the hypothesis.	Low-medium	Performance and classifications derived from this ER pathway model of 1812 substances were compared to high quality *in vitro* and *in vivo* data for specific reference chemicals. Depending on the combination of tests used, this prediction model generally achieved accuracies of 84 to 100% as compared to reference chemicals or the US EPA's Endocrine Disruptor Screening Program (EDSP) Tier 1 guideline *in vitro* tests and uterotrophic assay. These results verify the applicability of the ER pathway model as an IATA for identification of potential endocrine disruption for a wide range of chemicals that may act as agonists of estrogen receptor activation.
Overall uncertainty of the IATA.	Low	Uncertainty associated with the use of the IATA is judged to be low. Multiple HT assays are used along the estrogen receptor signalling pathway. This set of biochemical and cell-based *in vitro* assays relies on different technologies and probes different key events in the ER signalling pathway. Although the assays primarily use human proteins and/or cell types, the suite of 16 assays (see Annex I) include human, murine, and bovine ER binding assays and ER pathway interactions in a variety of human tissue types with some redundancy across species for some key events. The full ER pathway model accuracy was 84% to 93% when compared to reference chemicals and predicted results of EDSP Tier 1 guideline and other uterotrophic studies with 84% to 100% accuracy if inconclusive chemicals are excluded (Table 3). The performance of high-throughput assays and ER pathway model predictions demonstrates that these methods correctly identify active and inactive reference chemicals, provide a measure of relative ER agonist activity, and rapidly identify chemicals with potential endocrine bioactivities for additional screening and testing.

Accounting for uncertainty in in vitro HTS Assays and Pathway Model Analysis: In the analysis of the HTS assays, statistical methods have been developed to establish uncertainty bounds around potency and efficacy values. These statistical methods involve resampling the data and refitting the concentration response curves thousands of times to quantitatively estimate the uncertainty. Concentration-response parameters such as potency and efficacy are extracted from HTS data using nonlinear regression, and models and analyses built from these parameters are used to predict *in vivo* and *in vitro* toxicity of thousands of chemicals. An explicit method for accounting for uncertainty in potency, efficacy and binary activity (active vs. inactive) has been developed (Watt and Judson, 2018). This study used nonparametric bootstrap resampling to calculate experimental uncertainties in concentration-response parameters from each of the *in vitro* HTS assays. Then these individual uncertainties are propagated through the integrated computational model to produce confidence intervals in the ER pathway model scores for agonist activity. Specifically, for each *in vitro* assay and concentration response curve used as input in the model, one thousand bootstrap replicates were generated to obtain a distribution of fit

parameters, i.e. model selection, potency estimates, and activity calls. The uncertainty associated with the concentration response in each assay was propagated to the ER pathway model score (i.e., AUC values) by rerunning the full model 1000 times for each chemical enabling the calculation of confidence intervals for each chemical (Figure 7).

At the individual chemical-assay level, curves with high variability are flagged for manual inspection or retesting, focusing subject-matter-expert time on results that need further input. This work improves the confidence of predictions made using HTS data, increasing the ability to use this data in risk assessment. Also, by limiting the manual inspection to only those chemicals with large variability and quantifying which curves are contributing to that variability, subject-matter-expert time is optimised for studying only the most difficult examples.

Figure 7. ER pathway model results for chemicals with an AUC (Agonist) value > 0.1 (Watt and Judson, 2018).



Point estimates for agonist (red) and assay interference node (blue) values are marked by circles for all AUC values with an upper 95% confidence interval > 0.1. Error bars indicate the 95% confidence interval obtained by bootstrap resampling.

5.5. Strategy and Integrated Conclusion

As described above, this case study presents an IATA which is an ITS for the identification of potential endocrine disruption through ER agonist activity by a substance. The DIP described here includes multiple *in vitro* assays based on a series of molecular events that typically occur in a nuclear receptor-mediated response. This model has been evaluated for possible use as an IATA using 4 to 16 *in vitro* HTS assays. The results of these ER high-throughput screening assays were integrated into a computational model that can discriminate ER agonist activity from assay-specific interference and cytotoxicity. Model

scores range from 0 (no activity) to 1 (activity of 17β-estradiol). As long as the combination of test methods employed in the DIP covers the MIE and multiple KEs in the pathway, the performance-based evaluation of the ER pathway model demonstrates that there is flexibility in the number and selection of *in vitro* HTS assays to be used to determine the ER agonist activity of a chemical as long as the assays meet the criteria described. For a positive result, the computational model requires concordant results for multiple assays and KEs. These results may then contribute to the weight of evidence analysis based on other scientifically relevant information on a chemical's potential for endocrine disruption.

In contrast to approaches which involve some degree of expert judgement, the benefit to using an IATA such as the integrated testing strategy presented here is that it is rule-based and can provide predictable outcomes that can either be used on their own or considered together with other sources of information. The IATA described here has been demonstrated to predict ER agonist activity of both *in vitro* and *in vivo* reference chemicals with concordance ranging from 84 – 93%. The results of this performance-based evaluation of the ER pathway model demonstrate the accuracy, sensitivity and specificity of this IATA for screening of chemicals for ER agonist activity. The results of the analysis of this IATA gives scientific support for the use of these HTS assays as alternatives for existing OECD TGs, for potential use in regulatory decisions related to estrogenic activity.

6. Acknowledgements

The authors would like to acknowledge Drs. Anna Lowit, Stan Barone and Office of Science Coordination Policy (OSCP, OCSPP, US EPA) colleagues including Drs. Ronnie Bever, Seema Schappelle, Sharlene Matten, Andrea Kirk as well as Mr. Kristan Markey for their careful review of this document. Special thanks to Drs. Stacie Flood and Keith Houck for their efforts to compile the detailed assay information in Annex I.

7. References

Auld, D. S., Thorne, N., Nguyen, D. T., and Inglese, J. (2008), "A specific mechanism for nonspecific activation in reporter-gene assays." ACS Chem. Biol. 3, 463–470.

Baell, J. B., and Holloway, G. A. (2010), "New substructure filters for removal of pan assay interference compounds (PAINS) from screening libraries and for their exclusion in bioassays." J. Med. Chem. 53, 2719–2740.

Browne, P., et al. (2017), "Application of Adverse Outcome Pathways to U.S. EPA's Endocrine Disruptor Screening Program", Environmental Health Perspectives, vol 125 (9): 1-11. https://doi.org/10.1289/EHP1304.

Browne, P., et al. (2015), "Screening Chemicals for Estrogen Receptor Bioactivity Using a Computational Model", Environmental Science & Technology, vol 49 (14): 8804-14.

Bruns, R. F., and Watson, I. A. (2012), "Rules for identifying potentially reactive or promiscuous compounds." J. Med. Chem., 55: 9763–9772.

Canadian Environmental Protection Act (CEPA) (1999), part 3 Section 44(4): Hormone disrupting substances. http://laws-lois.justice.gc.ca/eng/acts/c-15.31/FullText.html [accessed 20 March 2019]

Colborn T, Clement C. (1992), "Chemically induced alterations in sexual and functional development: The wildlife/human connection". In: Advances in Modern Environmental Toxicology, Vol. 21. Mehlman MA, ed. Princeton, NJ: Princeton Scientific Publishing Co., Inc.

Dix, D.J., et al., (2007), "The ToxCast program for prioritizing toxicity testing of environmental chemicals." Toxicol Sci 95(1): 5- 12.

Dreier, D. et al., (2017), "Computational analysis of the ToxCast estrogen receptor agonist assays to predict vitellogenin induction by chemicals in male fish." Environmental Toxicology and Pharmacology. 53:177-183.

European Chemicals Agency and European Food Safety Agency (2018), "Guidance for the identification of endocrine disruptors in the context of Regulations (EU) No 528/2012 and (EC) No 1107/2009. EFSA Journal 2018;16(6):5311. https://doi.org/10.2903/j.efsa.2018.5311. ECHA-18-G-01-EN. [accessed 21 March 2019].

European Commission (2018), "Towards a comprehensive European Union framework on endocrine disruptors", Communication from the Commission to the European Parliament, the Council, the European Economic and Social Committee and the Committee of the Regions. COM(2018) 734 final. http://ec.europa.eu/transparency/regdoc/rep/1/2018/EN/COM-2018-734-F1-EN-MAIN-PART-1.PDF [accessed 04 December 2018].

Filer, D.L. (2017), "tcpl: the ToxCast pipeline for high-throughput screening data." Bioinformatics 33(4): 618-620. doi: 10.1093/bioinformatics/btw680.

Gore, A. C., et al. (2015), EDC-2: "The Endocrine Society's second scientific statement on endocrine-disrupting chemicals". Endocr Rev 36(6):E1–E150, PMID: 26544531, https://doi.org/10.1210/er.2015-1010.

Hotchkiss, A.K., et al. (2008), "Fifteen years after "Wingspread" – Environmental endocrine disrupters and human and wildlife health: Where we are today and where we need to go". Toxicol Sci 105(2):235–259, PMID: 18281716, https://doi.org/10.1093/toxsci/kfn030.

Judson et al. (2017), "On selecting a minimal set of *in vitro* assays to reliably determine estrogen agonist activity". Regulatory Toxicology and Pharmacology 91: 39-49.

Judson, R.S., et al. (2015), "Integrated model of chemical perturbations of a biological pathway using 18 *in vitro* high-throughput screening assays for the estrogen receptor". Toxicol Sci 148(1):137–154, PMID: 26272952, https://doi.org/10.1093/toxsci/kfv168.

Judson, R.S., et al. (2010), "*In vitro* screening of environmental chemicals for targeted testing prioritization: the ToxCast project." Environ. Health Perspect. 118, 485–492.

Kavlock, R.J., et al., (2012), "Update on EPA's ToxCast program: providing high throughput decision support tools for chemical risk management." Chem Res. Toxicol. 25, 1287–1302.

Kavlock, R.J., et al. (1996), "Research needs for the risk assessment of health and environmental effects of endocrine disruptors: A report of the U.S. EPA-sponsored workshop." Environ Health Perspect 104:715–740, PMID: 8880000, https://doi.org/10.2307/3432708.

Kleinstreuer, N.C., et al. (2016), "A curated database of rodent uterotrophic bioactivity." Environ Health Perspect 124(5):556–562, PMID: 26431337, https://doi.org/10.1289/ehp.1510183.

Miller, J. R., Thanabal, V., Melnick, M. M., Lall, M., Donovan, C., Sarver, R. W., Lee, D. Y., Ohren, J., and Emerson, D. (2010), "The use of biochemical and biophysical tools for triage of highthroughput screening hits - A case study with Escherichia coli phosphopantetheine adenylyltransferase". Chem. Biol. Drug Des. 75, 444–454.

OECD (2007). OECD Test No. 440. Uterotrophic Bioassay in Rodents: A Short-Term Screening Test for Oestrogenic Properties. OECD Guidelines for the Testing of Chemicals, Section 4: Health Effects. Available: https://doi.org/10.1787/9789264067417-en.

OECD (2015a), OECD Test No. 455: Performance-Based Test Guideline for Stably Transfected Transactivation *In vitro* Assays to Detect Estrogen Receptor Agonists and Antagonists, OECD Publishing, Paris. http://dx.doi.org/10.1787/9789264243040-en

OECD (2015b), OECD Test No. 493: Performance-Based Test Guideline for Human Recombinant Estrogen Receptor (hrER) *In vitro* Assays to Detect Chemicals with ER Binding Affinity, OECD Publishing, Paris. http://dx.doi.org/10.1787/9789264242623-en

OECD (2012a), "Conceptual framework for testing and assessment of endocrine disrupters", in Guidance document on standardized test guidelines for evaluating chemicals for endocrine disruption (GD150), OECD Publishing, Paris, https://www.oecd.org/officialdocuments/publicdisplaydocumentpdf/?cote=env/jm/mono%282012%2922&doclanguage=en.

OECD (2012b), OECD Test No. 457: Estrogen Receptor Transactivation Test Method for Identifying Estrogen Receptor Agonists and Antagonists, OECD Publishing, Paris. http://dx.doi.org/10.1787/9789264185395-en

Soto, A.M. and C. Sonnenschein. (2010), "Environmental causes of cancer: Endocrine disruptors as carcinogens." Nat Rev Endocrinol 6(7):364–371, PMID: 20498677, https://doi.org/10.1038/nrendo.2010.87.

Thorne, N., Auld, D. S., and Inglese, J. (2010), "Apparent activity in high-throughput screening: origins of compound-dependent assay interference." Curr. Opin. Chem. Biol. 14, 315–324.

US EPA (Environmental Protection Agency) (2015a), "Toxicity Forecaster (ToxCast) Data", https://www.epa.gov/chemical-research/toxicity-forecaster-toxcasttm-data [accessed 22 March 2018].

US EPA (2015b), Use of High Throughput Assays and Computational Tools; Endocrine Disruptor Screening Program; Notice of Availability and Opportunity for Comment. https://www.federalregister.gov/documents/2015/06/19/2015-15182/use-of-high-throughput-assays-and-computational-tools-endocrine-disruptor-screening-program-notice [accessed 13 September 2018].

US EPA (2015c), Transmittal of Meeting Minutes of the FIFRA Scientific Advisory Panel Meeting held on "Integrated Endocrine Bioactivity and Exposure-Based Prioritization and Screening." https://www.epa.gov/sites/production/files/2015-06/documents/120214minutes.pdf [accessed 13 September 2018].

US EPA (2014), Integrated Bioactivity and Exposure Ranking: A Computational Approach for the Prioritization and Screening of Chemicals in the Endocrine Disruptor Screening Program, Report Number. EPA-HQ-OPP-2014-0614-0003. https://www.regulations.gov/document?D=EPA-HQ-OPP-2014-0614-0003 [accessed 23 March 2018].

US EPA (2002), Summary of the Federal Food, Drug, and Cosmetic Act 21 U.S.C. §301 et seq. https://www.epa.gov/laws-regulations/summary-federal-food-drug-and-cosmetic-act [accessed 20 March 2019].

Watt, E.D. and R.S. Judson (2018), "Uncertainty Quantification in ToxCast High Throughput Screening," PLoS One. 2018; 13(7): e0196963. https://doi.org/10.1371/journal.pone.0196963

WHO (World Health Organization) (2002), "Global assessment of the state-of-the science of endocrine disruptors". http://www.who.int/ipcs/publications/new_issues/endocrine_disruptors/en/ [accessed 18 March 2018].

WHO/UNEP (United Nations Environmental Programme). (2012). "State of the science of endocrine disrupting chemicals – 2012". http://www.who.int/ceh/publications/endocrine/en/ [accessed 18 March 2018].

Annex I. Detailed assay descriptions in OECD GD211 Format

Please refer to the separate publication for full Annex I

ENV/JM/MONO(2019)28/ANN1

Annex II. Curated reference chemical lists.

Table AII-1. *In vitro* Estrogen Receptor (ER) Agonist Reference Chemicals

CASRN	Chemical name	Agonist potency [a]	ER pathway model score
57-63-6	17α-Ethinyl estradiol	strong	1
84-16-2	meso-Hexestrol	strong	0.99
56-53-1	Diethylstilbestrol (DES)	strong	0.94
50-28-2	17β-Estradiol	strong	0.94
57-91-0	17α-Estradiol	moderate	1.06
53-16-7	Estrone	moderate	0.81
140-66-9	4-tert-Octylphenol	moderate	0.39
446-72-0	Genistein	weak	0.54
77-40-7	Bisphenol B	weak	0.49
80-05-7	Bisphenol A	weak	0.45
486-66-8	Daidzein	weak	0.44
521-18-6	5α-Dihydrotestosterone	weak	0.40
789-02-6	o,p'-DDT	weak	0.39
599-64-4	4-Cumylphenol	weak	0.38
143-50-0	Kepone	weak	0.17
58-18-4	17α-Methyltestosterone	very weak	0.50
520-36-5	Apigenin	very weak	0.31
72-43-5	Methoxychlor	very weak	0.25
520-18-3	Kaempferol	very weak	0.25
85-68-7	Butylbenzyl phthalate	very weak	0.18
480-40-0	Chrysin	very weak	0.13
60168-88-9	Fenarimol	very weak	0.11
104-40-5	p-n-Nonylphenol	very weak	0.1
120-47-8	Ethylparaben	very weak	0.1
72-55-9	p,p'-DDE	very weak	0.1
84-74-2	Di-n-butyl phthalate	very weak	0.03
115-32-2	Dicofol	very weak	0
117-81-7	Diethylhexyl phthalate	very weak	0
52-86-8	Haloperidol	inactive	0.01
52-01-7	Spironolactone	inactive	0
50-22-6	Corticosterone	inactive	0
13311-84-7	Flutamide	inactive	0
1912-24-9	Atrazine	inactive	0
32809-16-8	Procymidone	inactive	0
330-55-2	Linuron	inactive	0
50-55-5	Reserpine	inactive	0
52806-53-8	Hydroxyflutamide	inactive	0
57-30-7	Phenobarbital Sodium	inactive	0
65277-42-1	Ketoconazole	inactive	0
66-81-9	Cycloheximide	inactive	0

a: Reference chemical potency, determined by concentration required to elicit 50% of the maximal response (AC50), in low throughput *in vitro* ER assays. More details on each chemical can be found through the hyperlinked CASRN. Strong = AC50 < 0.0001 µM, moderate = AC50 <0.1 µM, weak = AC50 < 1 µM, very weak = all other activities, and inactive = no detected activity.

Table AII-2. *In vivo* Estrogen Receptor (ER) Agonist Reference Chemicals with at Least two Independent Active or Inactive Guideline-like Uterotrophic Studies (Browne et al., 2015)

CASRN	Chemical name	Active [a]	Inactive [a]	Bioactivity	ER pathway model score
57-91-0	17α-Estradiol	2	0	active	1.06
57-63-6	Ethinyl Estradiol	59	0	active	1
56-53-1	Diethylstilbestrol (DES)	8	1	active	0.94
50-28-2	Estradiol	25	0	active	0.94
474-86-2	Equilin	2	0	active	0.82
53-16-7	Estrone	9	0	active	0.81
50-27-1	Estriol	4	0	active	0.79
72-33-3	Mestranol	3	0	active	0.74
17924-92-4	Zearalenone	4	0	active	0.71
1478-61-1	Bisphenol AF	4	0	active	0.55
446-72-0	Genistein	27	1	active	0.54
68-22-4	Norethindrone	2	0	active	0.52
58-18-4	Methyltestosterone	3	0	active	0.50
77-40-7	Bisphenol B	2	0	active	0.49
80-05-7	Bisphenol A	37	6	active	0.45
104-43-8	4-Dodecylphenol	3	0	active	0.41
521-18-6	Dihydrotestosterone	3	0	active	0.4
131-55-5	Benzophenone-2	6	0	active	0.40
140-66-9	4-(1,1,3,3-Tetramethylbutyl)phenol	3	1	active	0.39
789-02-6	o,p'-DDT	15	1	active	0.39
599-64-4	p-Cumylphenol	2	0	active	0.38
5153-25-3	Benzoic acid, 4-hydroxy-, 2-ethylhexyl ester	2	0	active	0.37
80-46-6	4-(1,1-Dimethylpropyl)phenol	4	0	active	0.28
131-56-6	2,4-Dihydroxybenzophenone	3	0	active	0.27
80-09-1	Bisphenol S	2	0	active	0.26
72-43-5	Methoxychlor	18	1	active	0.25
94-26-8	Butylparaben	8	2	active	0.25
98-54-4	p-tert-Butylphenol	2	0	active	0.16
104-40-5	Nonylphenol	5	4	active	0.10
556-67-2	Octamethylcyclotetrasiloxane	3	0	active	0
520-18-3	Kaempferol	0	3	inactive	0.25
84-74-2	Dibutyl phthalate	0	2	Inactive	0.03
84-61-7	Dicyclohexyl phthalate	0	2	inactive	0.02
84-75-3	Dihexyl phthalate	0	2	inactive	0.01
51630-58-1	Fenvalerate	0	2	inactive	0.01
103-23-1	Bis(2-ethylhexyl)hexanedioate	0	2	inactive	0
117-81-7	Bis(2-ethylhexyl)phthalate	0	2	inactive	0
1461-22-9	Tributylchlorostannane	0	2	inactive	0
1912-24-9	Atrazine	0	2	inactive	0
61-82-5	Amitrole	0	2	inactive	0
84-66-2	Diethyl phthalate	0	2	inactive	0
87-86-5	Pentachlorophenol	0	2	inactive	0
99-96-7	4-Hydroxybenzoic acid	0	2	inactive	0

a: The numbers of guideline-like active and inactive study results are reported for each chemical. More details on each chemical can be found through the hyperlinked CASRN.

Annex III. The Possible Combinations of 7 or Fewer Assays that Can Be Used in this IATA

This table shows all subset models with 7 or fewer assays with at least 94% balanced accuracy (BA) from all chemicals, and the *in vitro* and *in vivo* reference chemical sets.

				Binding			Dimerization								Transcription		Translation		Proliferation
Assays	BA (all chemicals)	BA (reference chemicals in vitro)	BA (reference chemicals in vivo)	NVS_NR_bER	NVS_NR_hER	NVS_NR_mERa	OT_ER_ERaERa_0480	OT_ER_ERaERa_1440	OT_ER_ERaERb_0480	OT_ER_ERaERb_1440	OT_ER_ERbERb_0480	OT_ER_ERbERb_1440	OT_ERa_EREGFP_0120	OT_ERa_EREGFP_0480	ATG_ERa_TRANS_up	ATG_ERE_CIS_up	TOX21_ERa_BLA_Agonist_ratio	TOX21_ERa_LUC_BG1_Agonist	ACEA_T47D_80hr_Positive
4	0.95	0.95	0.94		Y					Y					Y				Y
5	0.95	0.95	0.94		Y					Y	Y				Y				Y
6	0.95	0.95	0.94		Y	Y			Y	Y					Y				Y
6	0.95	0.95	0.94		Y	Y					Y	Y			Y				Y
6	0.95	0.95	0.94	Y	Y			Y						Y	Y				Y
6	0.96	0.95	0.94	Y	Y					Y				Y	Y				Y
6	0.96	0.95	0.94	Y	Y							Y		Y	Y				Y
6	0.96	0.95	0.94	Y	Y		Y							Y	Y				Y
7	0.95	0.95	0.94	Y	Y	Y		Y						Y	Y				Y
7	0.95	0.95	0.94	Y	Y			Y	Y					Y	Y				Y
7	0.95	0.95	0.94	Y	Y					Y	Y			Y	Y				Y
7	0.96	0.95	0.94	Y	Y		Y			Y				Y	Y				Y
7	0.96	0.95	0.94	Y	Y		Y					Y		Y	Y				Y
7	0.96	0.95	0.94	Y	Y					Y		Y		Y	Y				Y
7	0.96	0.95	0.94	Y	Y	Y				Y				Y	Y				Y
7	0.96	0.95	0.94	Y	Y				Y	Y				Y	Y				Y
7	0.96	0.95	0.94	Y	Y	Y						Y		Y	Y				Y
7	0.96	0.95	0.94	Y	Y				Y			Y		Y	Y				Y
7	0.96	0.95	0.94	Y	Y	Y	Y							Y	Y				Y

The results of the subset analysis demonstrated that there were many subsets of assays that performed at levels comparable to the full 16-assay model, including some that included as few as 4 assays. This table shows all subset models with 7 or fewer assays that achieve BA ≥94% for the full chemical set and for the *in vitro* and *in vivo* subsets. Almost all of these models use the cell-free human and bovine radioligand binding assays, the ATG TRANS transactivation assay and the ACEA cell proliferation assay. None of the subset models use the Tox21 transactivation assays. The main difference between these models is which of the Odyssey Thera dimerisation assays used. Green squares with a Y = those used in the subset; remaining squares are those not used in the subset.

Annex IV. Main Scientific Papers

Browne, P., et al. (2015), "Screening Chemicals for Estrogen Receptor Bioactivity Using a Computational Model", Environmental Science & Technology, vol 49 (14): 8804-14.

This publication provided description of the performance-based evaluation of the ER pathway model and general description of the reference chemicals used.

Kleinstreuer, N.C., et al. (2016) "A curated database of rodent uterotrophic bioactivity." Environ Health Perspect 124(5):556–562, PMID: 26431337, https://doi.org/10.1289/ehp.1510183.

This publication provided detailed information on the curation of the *in vivo* reference chemicals used in the performance evaluation of the ER pathway model.

Judson, R.S., et al. (2015), "Integrated model of chemical perturbations of a biological pathway using 18 *in vitro* high-throughput screening assays for the estrogen receptor". Toxicol Sci 148(1):137–154, PMID: 26272952, https://doi.org/10.1093/toxsci/kfv168.

This publication provided the basis of the ER pathway model and provides a general overview of the model.

Judson et al. (2017), "On selecting a minimal set of *in vitro* assays to reliably determine estrogen agonist activity". Regulatory Toxicology and Pharmacology 91: 39-49.

This publication describes the further work which allowed the 16-assay ER pathway model to be refined to a subset of as few as 4 assays.

Watt, E.D. and R.S. Judson (2018), Uncertainty Quantification in ToxCast High Throughput Screening, PLoS One. 2018; 13(7): e0196963. https://doi.org/10.1371/journal.pone.0196963

This publication proposes an analysis of the uncertainty related to the ER pathway model.