Case Study on the Use of an Integrated Approach to Testing and Assessment for Identifying Estrogen Receptor Active Chemicals

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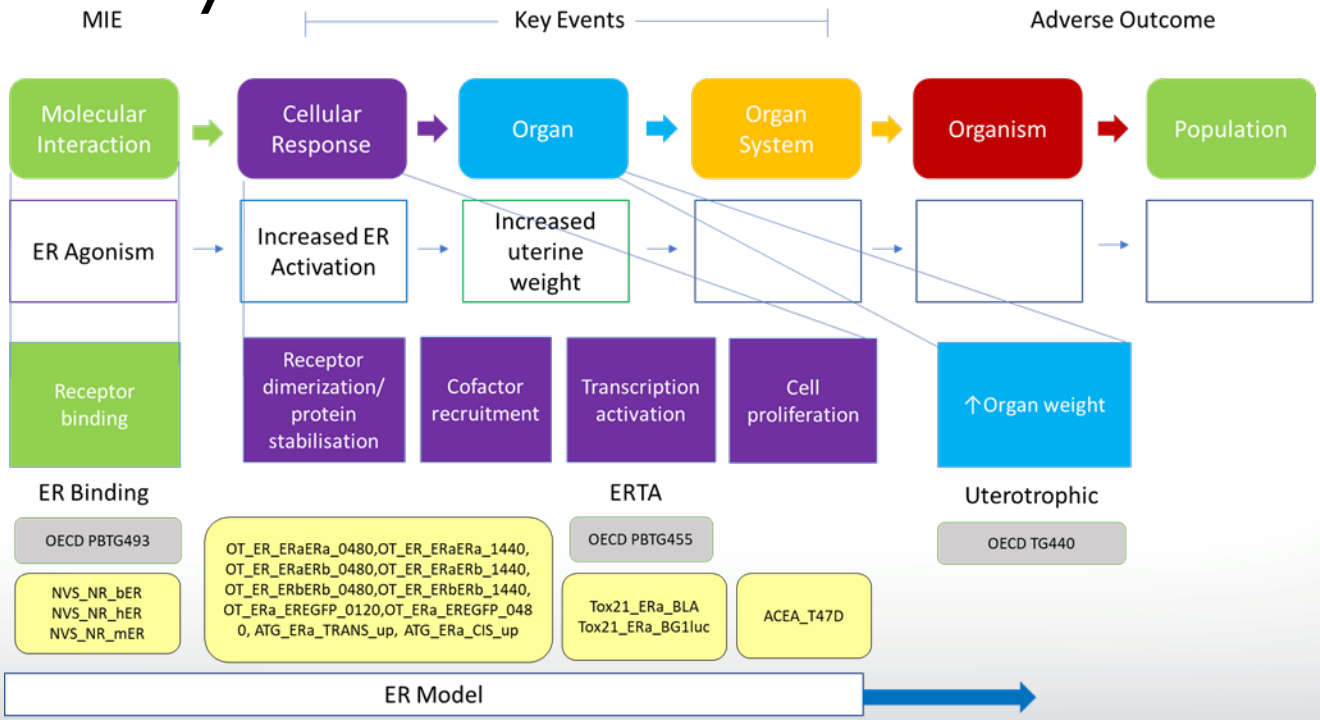
The views expressed in this presentation are that of the presenter and do not represent the views and/or policies of the US Environmental Protection Agency.

Intended Application

- The intended application of this IATA is for
  - screening of environmental chemicals based on their ER agonist activity
  - determining whether further evaluation of endocrine-related activity in higher tier in vivo tests (e.g., female pubertal assay, two generation reproductive toxicity study) is needed

Purpose

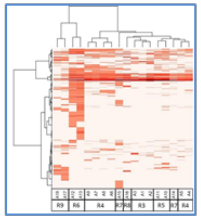
- To use a combination of 4 to 16 in vitro high throughput screening (HTS) assays and a computational model for estrogen receptor (ER) agonist activity, as an alternative to low and medium throughput in vitro and in vivo tests for ER activity.



Overall Approach

Internal

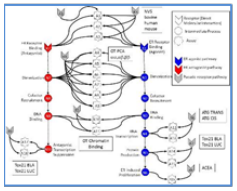
External



ER High-Throughput Screening Data

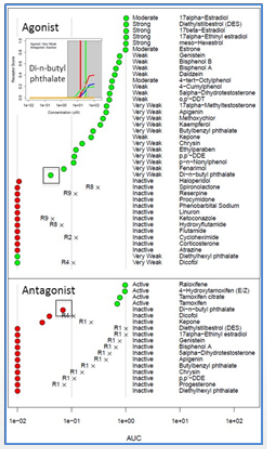
In vitro reference chemicals

OECD BETTER POLICIES FOR BETTER LIVES

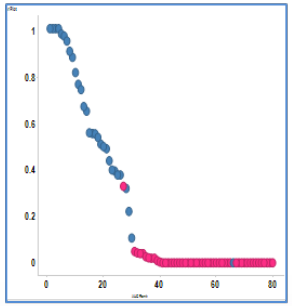


ER Pathway Computational Model

In vivo reference chemicals



Model Performance Evaluation



Validated Model for Chemical Screening

IATA Process

Run Assays
(Assays 1 - n, cytotoxicity)

- Choose at least 4 in vitro HTS assays that fit the criteria as described. Briefly, the assays chosen must probe diverse points in the ER pathway and use diverse assay reporting technologies and cell types.
- The possible combinations of 4 assays that can be used together are listed in Annex III. More than 4 assays can be chosen, in which case the results of all chosen assays must be modelled and the results reported. Concurrently evaluate a phenotypic response, cellular proliferation.

Import Data and Run Model

- The next step is to collect data from the assays into a format that the computational model's R-code can import and analyse. Ideally, this process will be performed automatically (electronically) to reduce the chance of user-input error.
- Although useful, advanced computational expertise is not necessary to run the model. For example, the formula used for the subset model analysis could be implemented in a spreadsheet so limited computational expertise required.

Generate Report

- The report should conform to the usual report format of executive summary, methods, results, and discussion. The report should include the raw data to allow the regulatory agencies to analyze the data themselves. It should also include summary tables with the AUC and AC50 values. Figures can be included when needed. The report should also include the results from the cytotoxicity assay.
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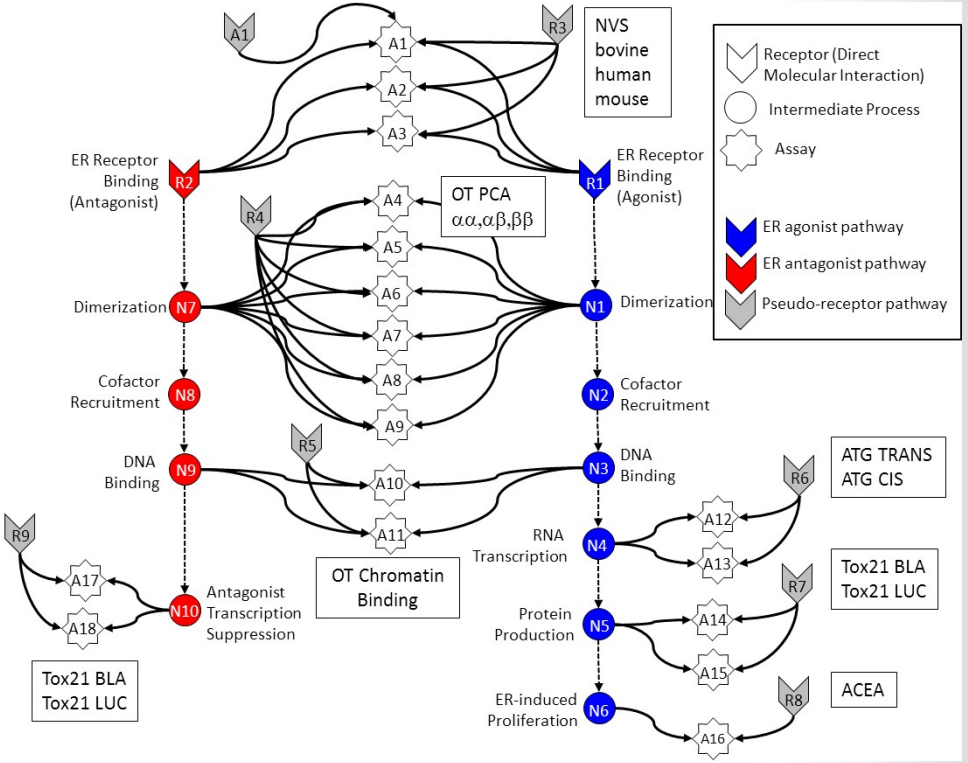
In Vitro Estrogen Receptor Model

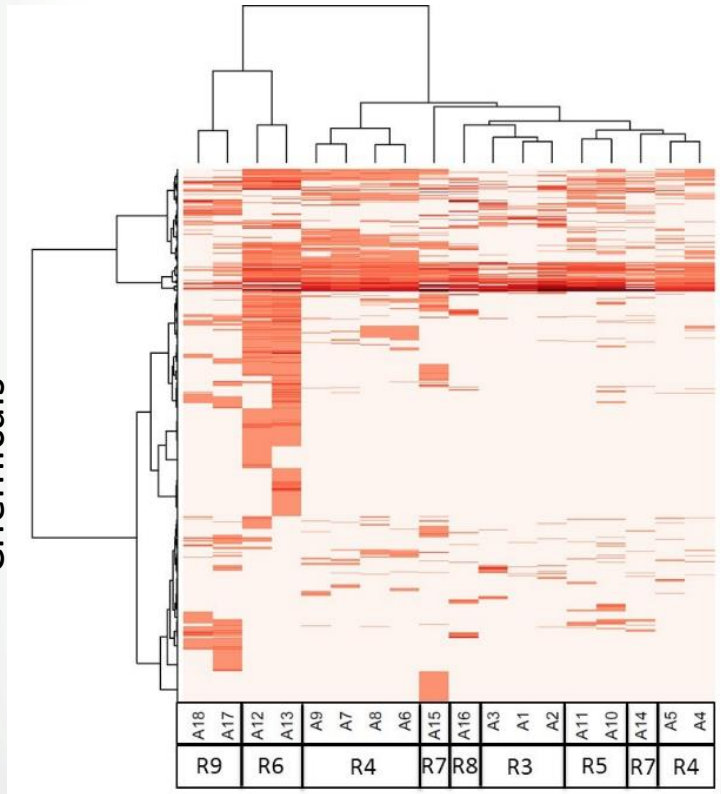
- **No *in vitro* assay is perfect**
  - Assay Interference
  - Noise
- **Use multiple assays per pathway**
  - Different technologies
  - Different points in pathway
- **Use model to integrate assays**
- **Evaluate model against reference chemicals**



Judson et al: "Integrated Model of Chemical Perturbations of a Biological Pathway Using 18 In Vitro High Throughput Screening Assays for the Estrogen Receptor" (EHP 2015)

All In vitro assays have false positives and negatives

Assays cluster by technology, suggesting technology-specific non-ER bioactivity

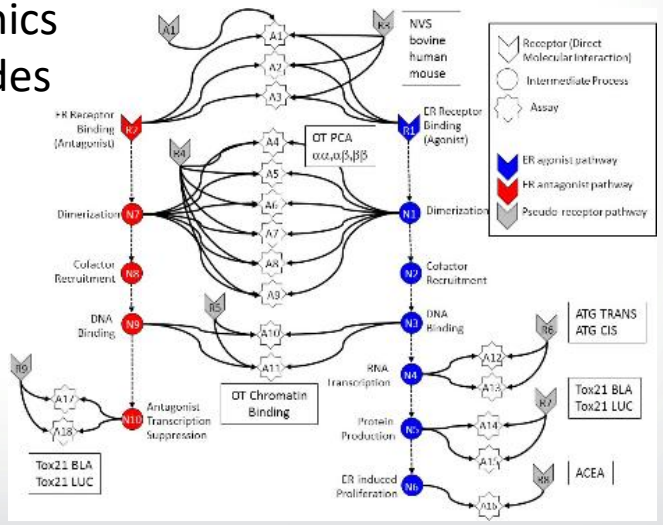


Much of this "noise" is reproducible

- "assay interference"
- Result of interaction of chemical with complex biology in the assay

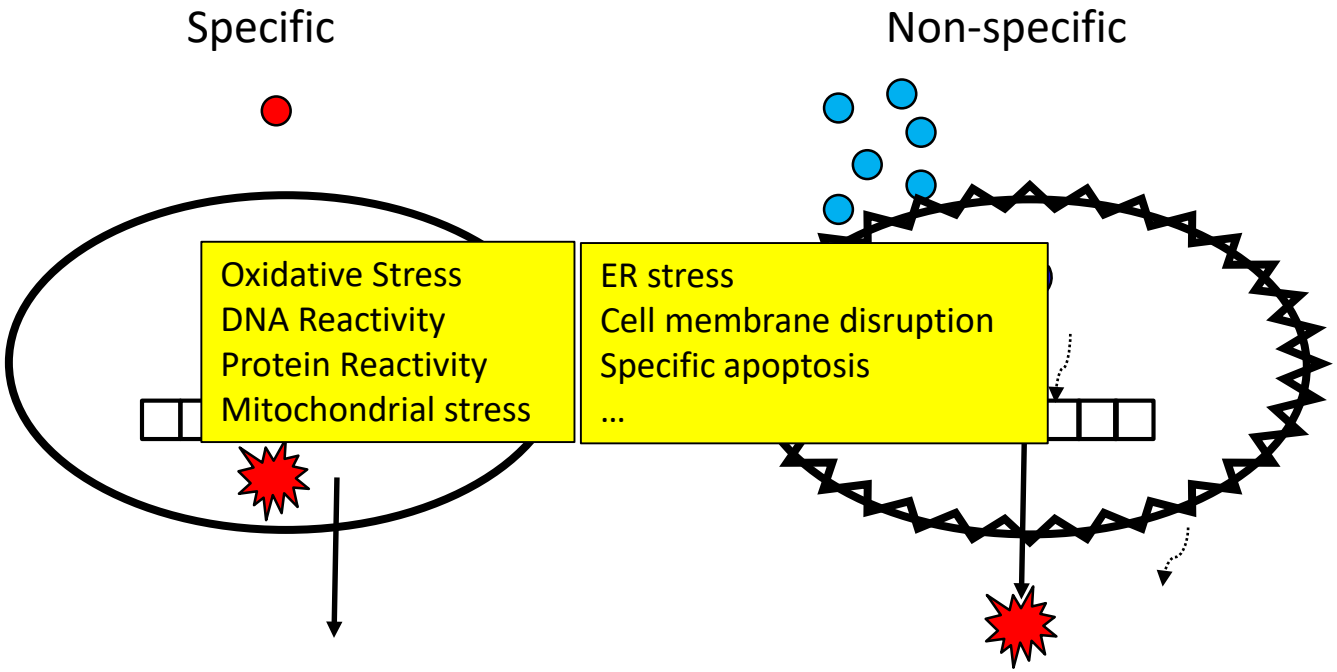
Chemical universe is structurally diverse

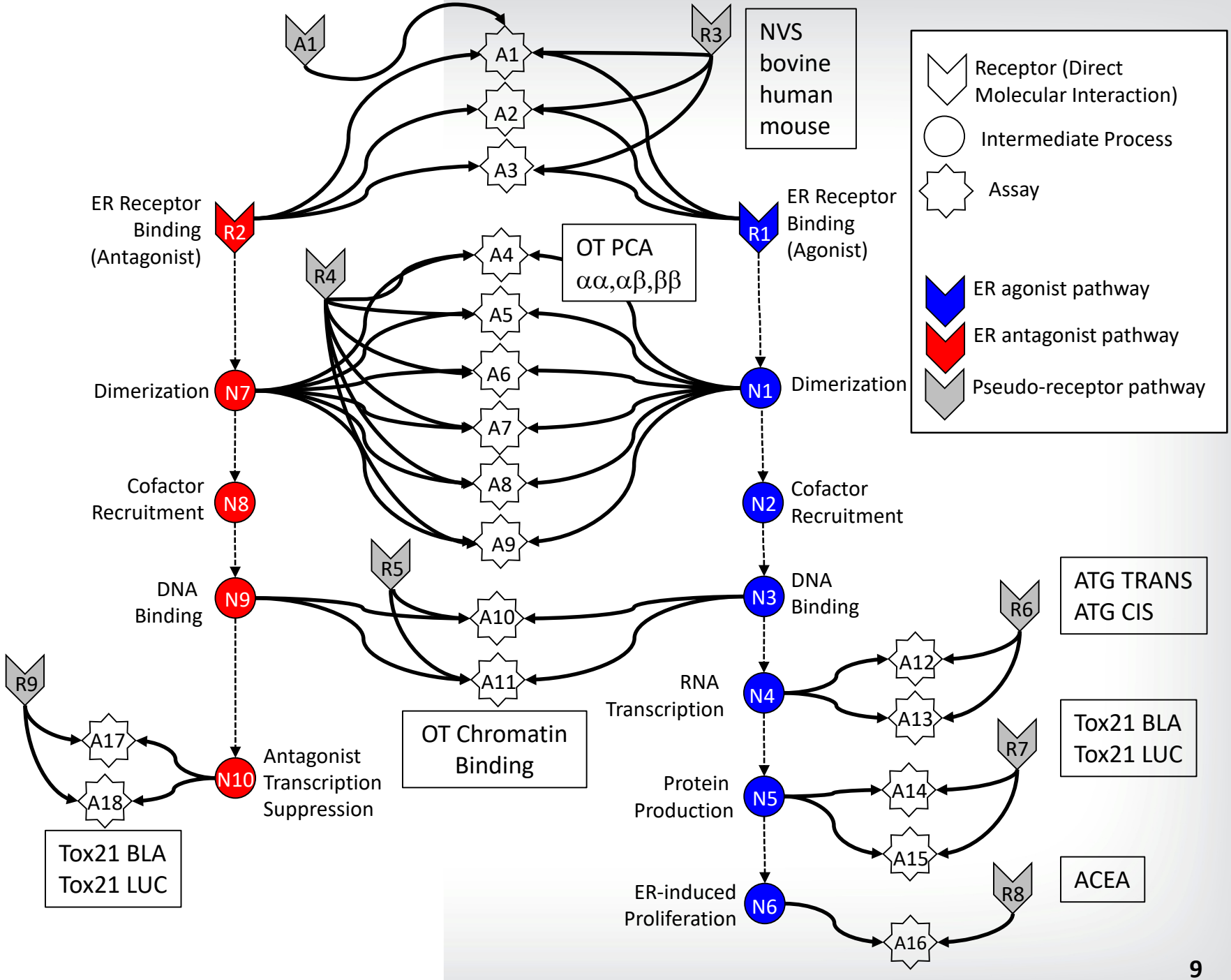
-Solvents
-Surfactants
-Intentionally cytotoxic compounds
-Metals
-Inorganics
-Pesticides
-Drugs

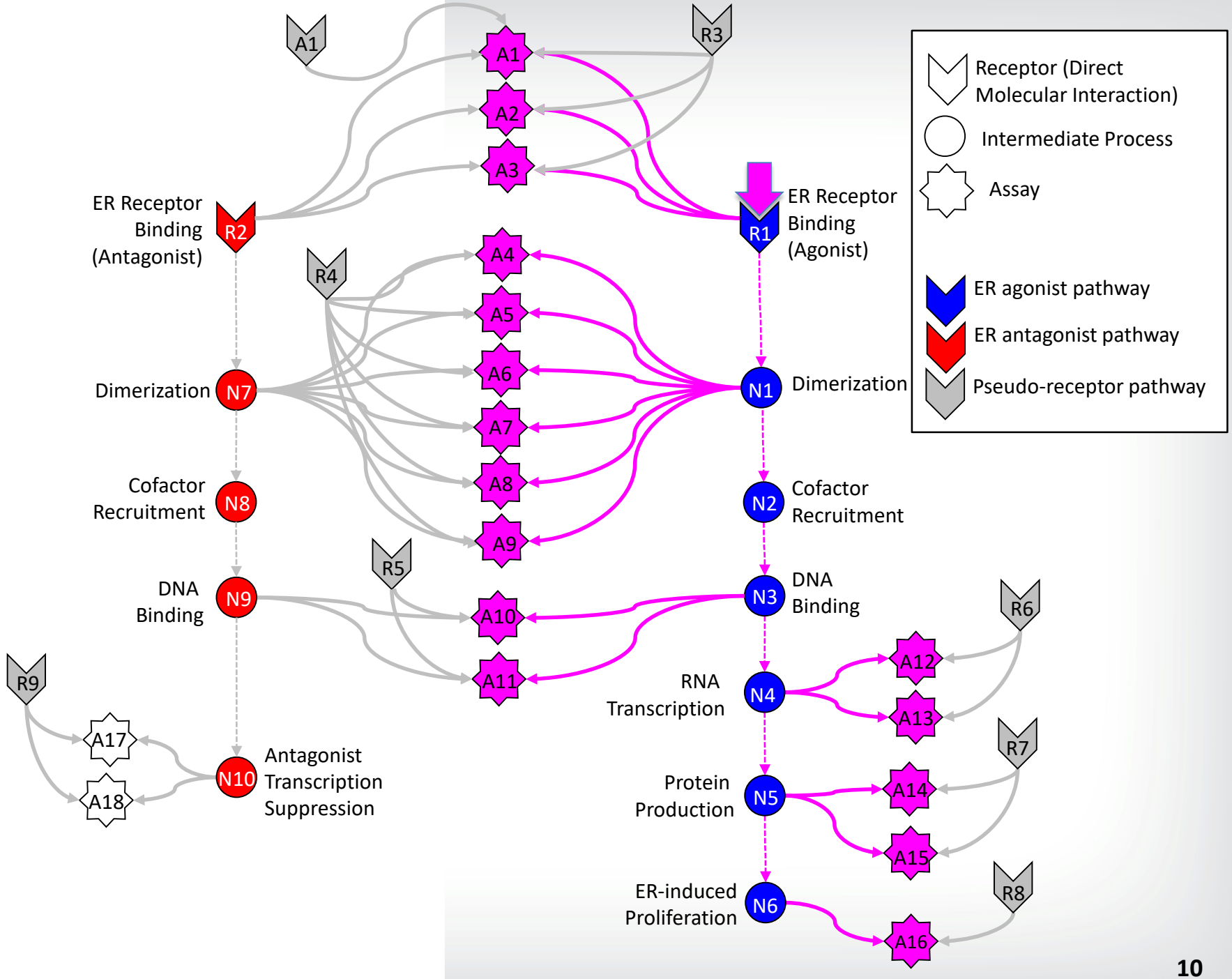


Judson et al: ToxSci (2015)

Schematic explanation of non-specific activity




NVS
bovine
human
mouse
Receptor (Direct Molecular Interaction)
Intermediate Process
Assay
ER agonist pathway
ER antagonist pathway
Pseudo-receptor pathway
A1
A2
A3
R1
R2
R3
ER Receptor Binding (Antagonist)
ER Receptor Binding (Agonist)
OT PCA
αα,αβ,ββ
A4
A5
A6
A7
A8
A9
R4
Dimerization
N7
N1
Dimerization
Cofactor Recruitment
N8
N2
Cofactor Recruitment
DNA Binding
N9
N3
DNA Binding
R5
A10
A11
OT Chromatin Binding
ATG TRANS
ATG CIS
R6
A12
A13
RNA Transcription
N4
Tox21 BLA
Tox21 LUC
R7
A14
A15
Protein Production
N5
ACEA
R8
A16
ER-induced Proliferation
N6
R9
A17
A18
N10
Antagonist Transcription Suppression
Tox21 BLA
Tox21 LUC


Receptor (Direct Molecular Interaction)
Intermediate Process
Assay
ER agonist pathway
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R1
ER Receptor Binding (Agonist)
R2
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R3
R4
R5
R6
R7
R8
R9
N1
Dimerization
N2
Cofactor Recruitment
N3
DNA Binding
N4
RNA Transcription
N5
Protein Production
N6
ER-induced Proliferation
N7
Dimerization
N8
Cofactor Recruitment
N9
DNA Binding
N10
Antagonist Transcription Suppression
A1
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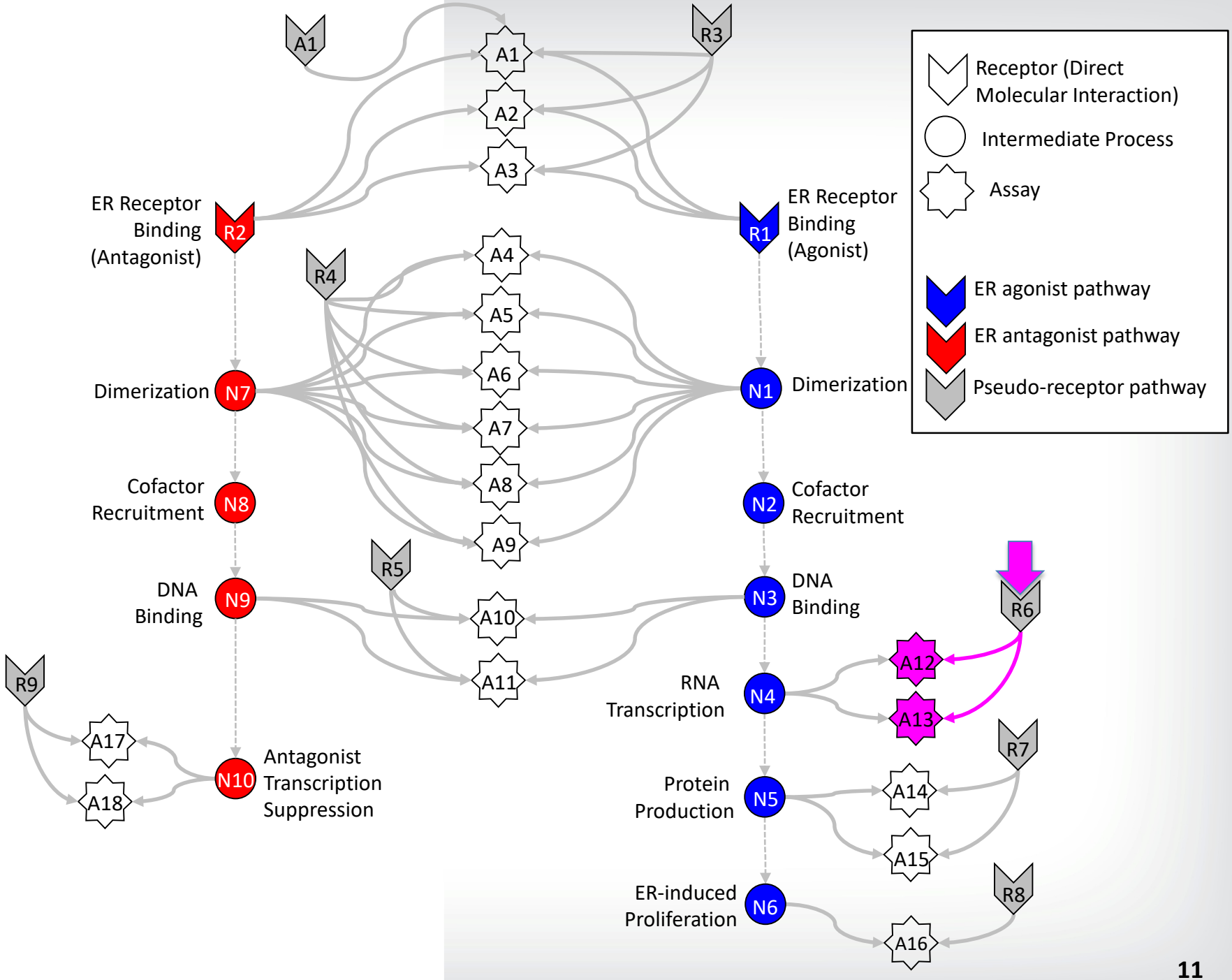
Receptor (Direct Molecular Interaction)
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ER agonist pathway
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Dimerization
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R1
R2
R3
R4
R5
R6
R7
R8
R9
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A14
A15
A16
A17
A18
N1
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N3
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N5
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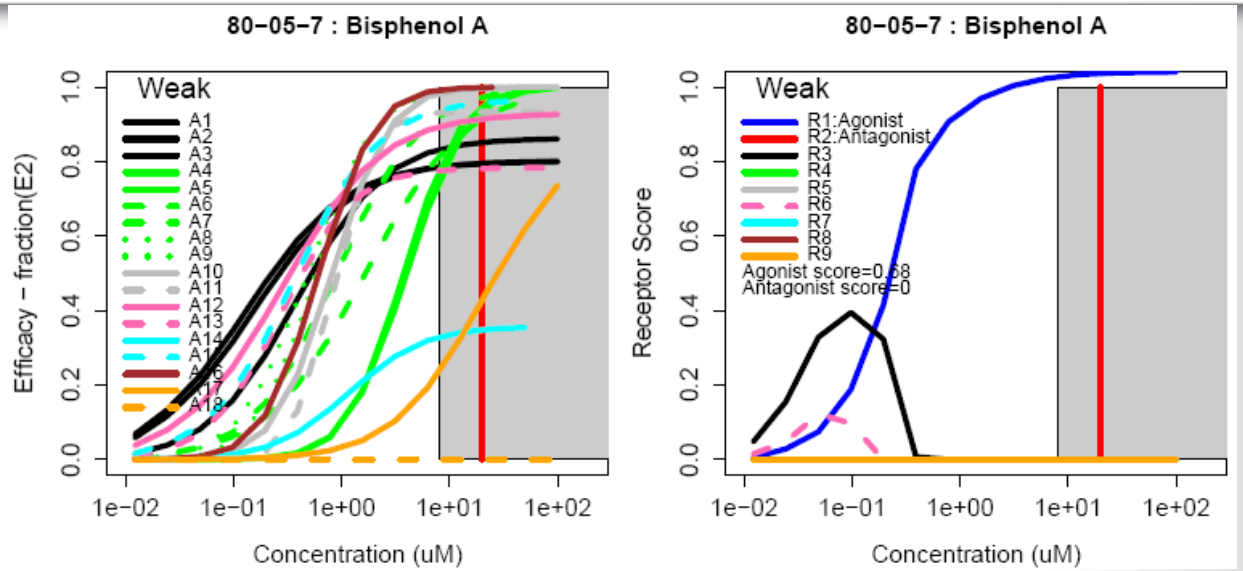
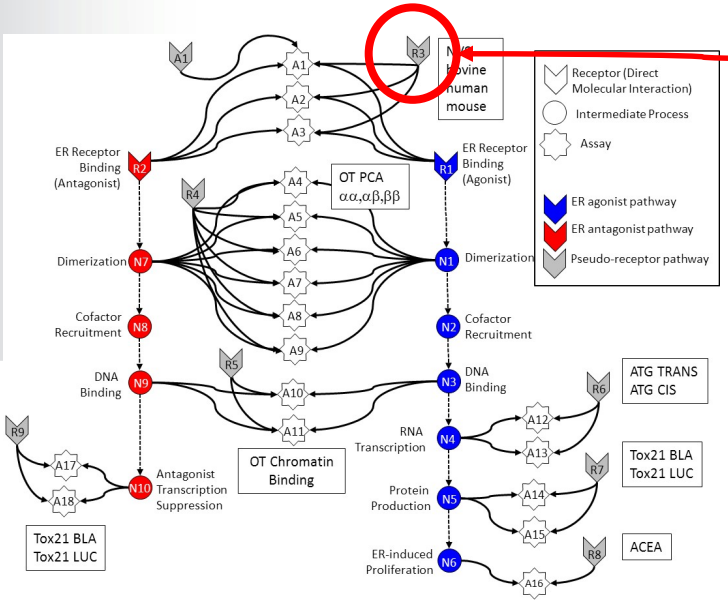
N9
N10

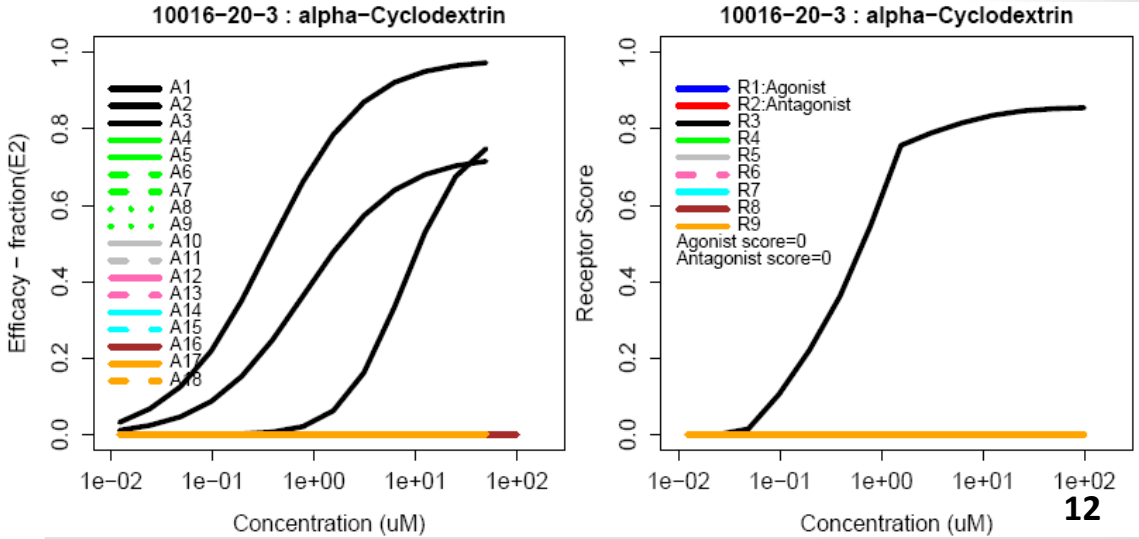
Example chemicals: Observe quantitative uncertainty

True Agonist





Assay Interference Example “R3”



Use of a Subset Model in the IATA

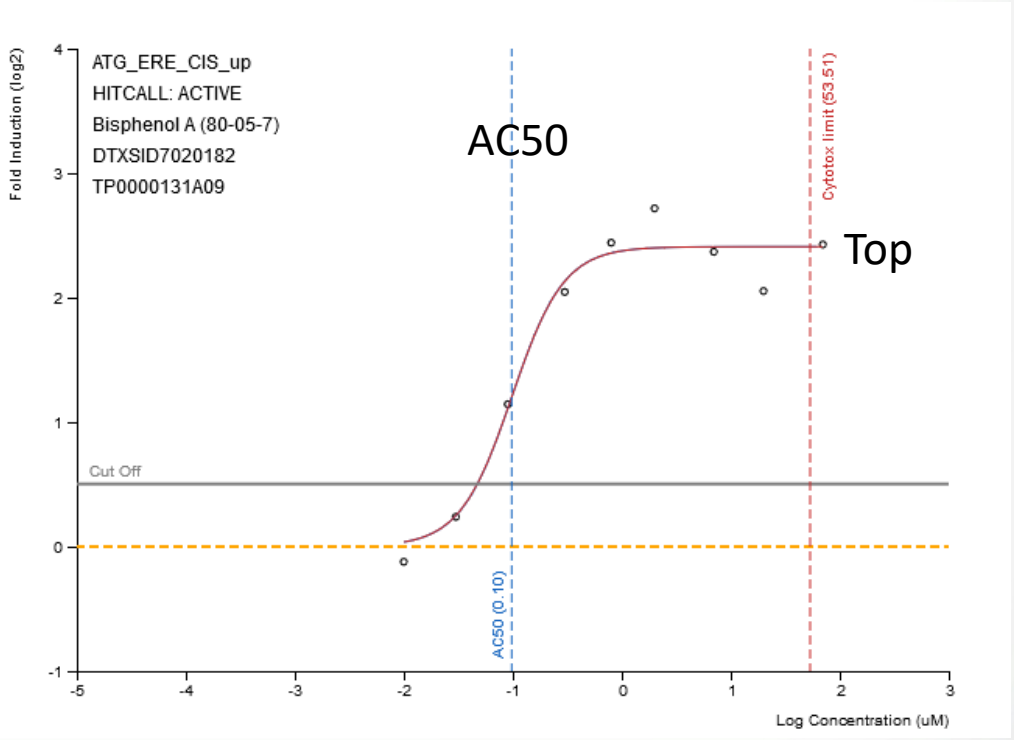
- The IATA has been clarified further to demonstrate that:
  - a subset of as few as 4 assays as an alternative approach to the current guideline
  - the subset of assays can be any assays that fit into specific criteria as described
    - interrogates different points on the ER pathway
    - incorporate different technologies
    - Includes diverse cell types
- Included in this case study is an annex that describes the 9 subset models with 7 or fewer assays that achieve ≥94% balanced accuracy for all chemicals and the in vitro and in vivo reference chemical sets. (Annex III).
- Further articulated the benefits of using a subset of assays in the IATA, specifically the flexibility it gives users by allowing the use of any assays that fit the described criteria.

Subset Model

- **Assume that the "full model" (16 agonist assays) provides acceptable prediction of ER agonist activity**
  - Model based on detailed biology
  - Validated against in vitro and in vivo reference chemicals
- **Build simple "subset models" using fewer assays**
  - Validate against the full model over 1811 chemicals and in vitro and in vivo reference chemicals
- **Input to the model are assay-chemical AUC values**

Subset Model Input

- **Inputs to model are chemical-assay AUC (area under the curve) values**
- **Run assay in concentration-response mode**
- **Fit to model (e.g. Hill model)**
- **Calculate AC50 and Top**
- **AUC = -log(AC50) x Top**



Example curve for Bisphenol A

Data from:

EPA CompTox Chemicals Dashboard

https://comptox.epa.gov

IATA Process

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(Assays 1 - n, cytotoxicity)

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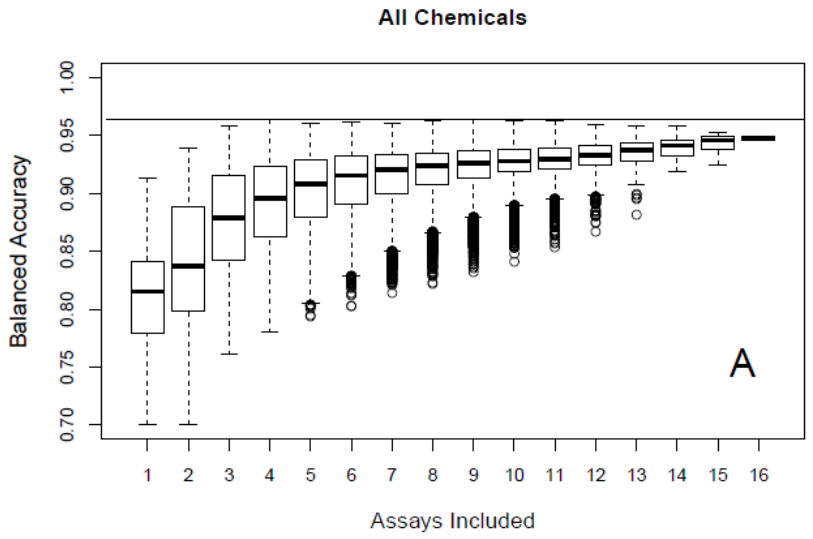
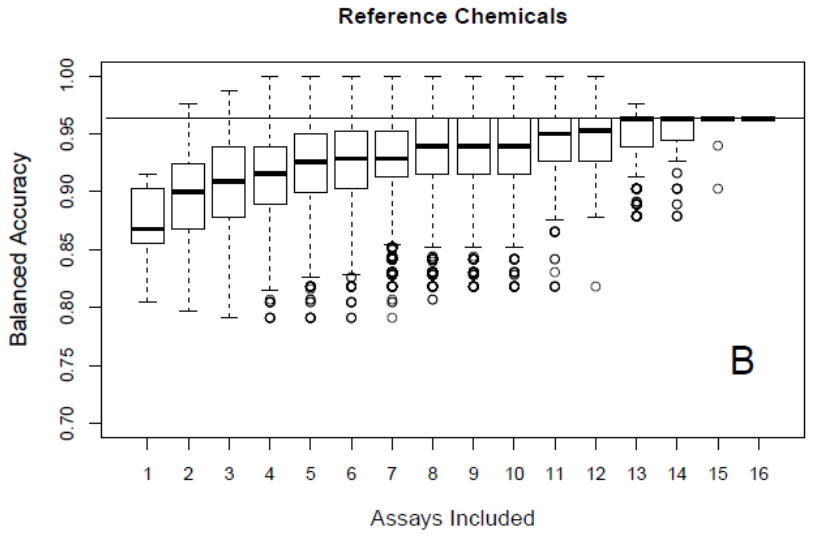
Building and Evaluating the Model

- **Step 1: For each set of assays (all combinations from 1 to 16), calculate the pathway AUC using the following formula …**
- $AUC_{subset}(chemical) = \sum_{i=1}^{N} c_i \times AUC(chemical, assay_i)$
- **Step 2: vary the weights (c_i) to minimize the squared difference between the subset pathway AUC and the full model AUC across all 1811 chemicals**
  - **This is a standard linear model inverse problem**
- **Calculate several statistics on each subset pathway model**
  - **Sensitivity, specificity, balanced accuracy for all chemicals**
  - **Sensitivity, specificity, balanced accuracy for in vitro reference chemicals**
  - **Sensitivity, specificity, balanced accuracy for in vivo reference chemicals**
  - **(balanced accuracy is average of sensitivity and specificity)**
- **Allow a user to select any model (i.e. any subset of assays) that provides high enough sensitivity, specificity, balanced accuracy across the 3 chemical group**

Statistical Results (1)



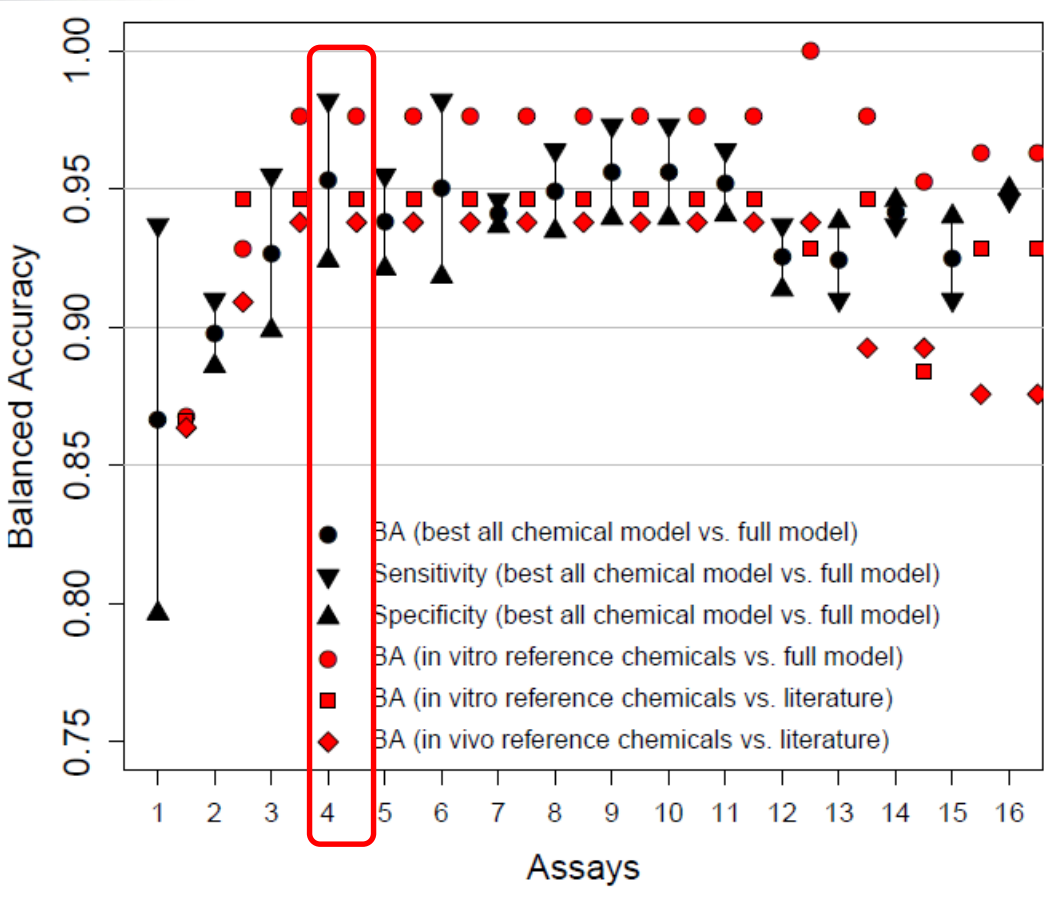


Boxplots show balanced accuracy for all models with a specified number of assays

- Top (all chemicals)
- Bottom (all reference chemicals, combined in vitro and in vivo)

Observe that with as few as 4 assays, there are subset models where reference chemical balanced accuracy is perfect and other subset models where all-chemical balanced accuracy is as high as it gets (93%)

Statistical Results (II)



Model that optimizes one parameter does not always optimize the other

This plot shows the statistics for the best overall model for a given number of assays

Example: best overall 4-assay model gives balanced accuracy of

- All chemicals: 0.955
- In vitro reference chemicals: 0.945
- In vivo reference chemicals: 0.94

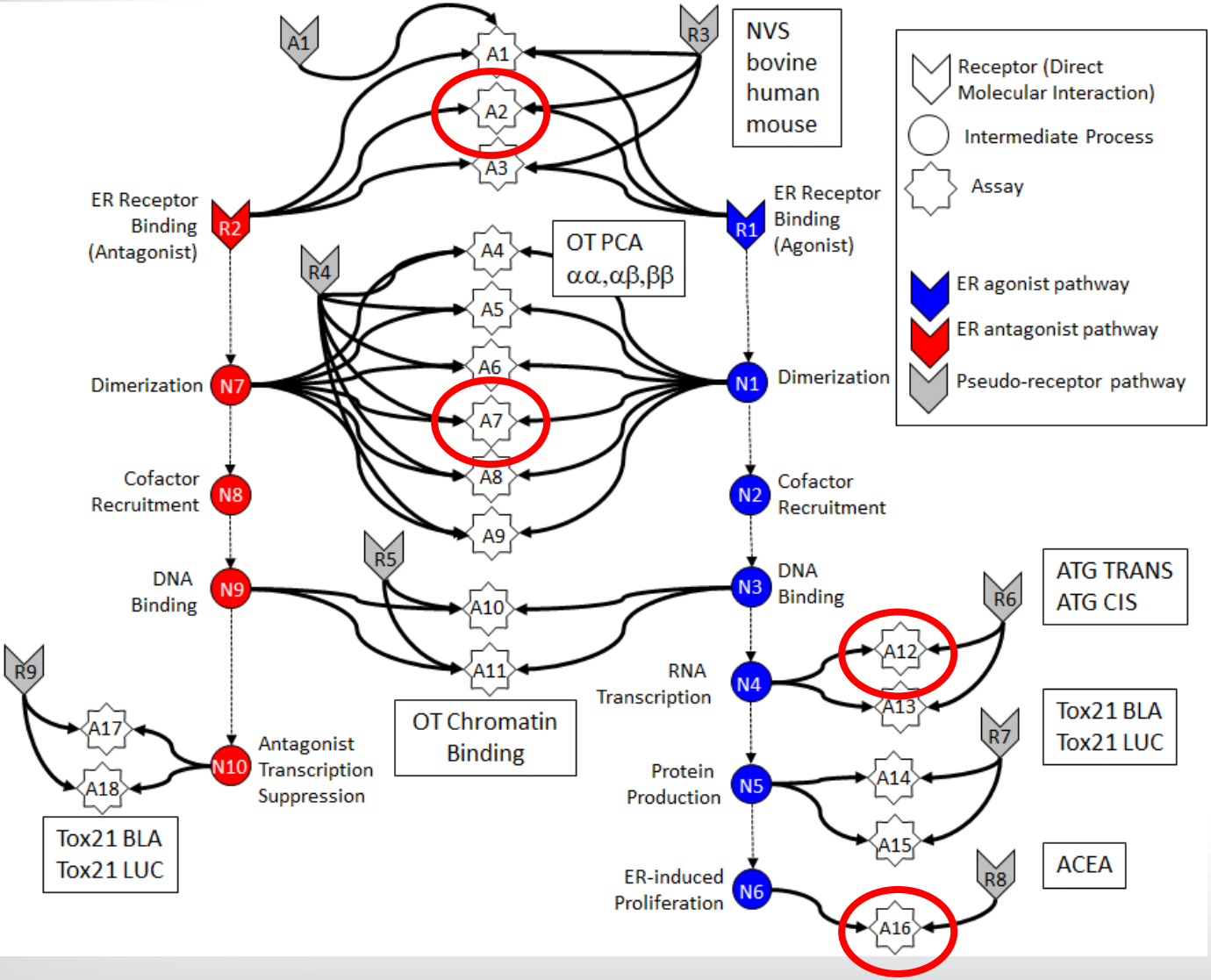
Sensitivity is higher than specificity (minimize false negatives)

Note that models with intermediate number of assays (4-11) perform better than those with more or fewer

What assays are used? (best assay sets)

Assays	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
Sensitivity (all chemicals)	0.94	0.91	0.95	0.98	0.95	0.98	0.95	0.96	0.97	0.97	0.96	0.94	0.91	0.94	0.91	0.95
Specificity (all chemicals)	0.80	0.89	0.90	0.92	0.92	0.92	0.94	0.93	0.94	0.94	0.94	0.91	0.94	0.95	0.94	0.95
BA (all chemicals)	0.87	0.90	0.93	0.95	0.94	0.95	0.94	0.95	0.96	0.96	0.95	0.93	0.92	0.94	0.92	0.95
BA (in vitro reference chemicals)	0.87	0.95	0.95	0.95	0.95	0.95	0.95	0.95	0.95	0.95	0.95	0.93	0.95	0.88	0.93	0.93
BA (in vivo reference chemicals)	0.86	0.91	0.94	0.94	0.94	0.94	0.94	0.94	0.94	0.94	0.94	0.94	0.89	0.89	0.88	0.88
BA (minimum)	0.86	0.90	0.93	0.94	0.94	0.94	0.94	0.94	0.94	0.94	0.94	0.93	0.89	0.88	0.88	0.88
Assay Selection																
NVS_NR_bER	0	0	0	0	0	0	0	1	1	1	1	1	1	1	1	1
NVS_NR_hER	0	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1
NVS_NR_mERa	0	0	0	0	1	1	0	1	1	1	1	1	1	1	1	1
OT_ER_ERaERa_0480	0	0	0	0	0	0	1	1	1	1	1	1	1	1	1	1
OT_ER_ERaERa_1440	0	0	0	0	1	0	1	1	1	0	0	1	1	1	1	1
OT_ER_ERaERb_0480	0	0	0	0	0	1	1	0	1	1	1	1	1	1	1	1
OT_ER_ERaERb_1440	0	0	1	1	0	1	0	0	0	1	1	1	1	1	1	1
OT_ER_ERbERb_0480	0	0	0	0	0	0	1	0	0	1	1	1	1	1	1	1
OT_ER_ERbERb_1440	0	1	0	0	0	0	0	0	0	0	1	1	1	1	1	1
OT_ERa_EREGFP_0120	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1
OT_ERa_EREGFP_0480	0	0	0	0	0	0	0	1	1	1	1	0	0	0	1	1
ATG_ERa_TRANS_up	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
ATG_ERE_CIS_up	0	0	0	0	0	0	0	0	0	0	0	0	1	1	1	1
TOX21_ERa_BLA_Agonist_ratio	0	0	0	0	0	0	0	0	0	0	0	0	1	1	1	1
TOX21_ERa_LUC_BG1_Agonist	0	0	0	0	0	0	0	0	0	0	0	1	1	1	1	1
ACEA_T47D_80hr_Positive	0	0	0	1	1	1	1	1	1	1	1	1	0	1	0	1

Where do these assays fall on the pathway?



Selected assays come from different points in the pathway and used different assay technologies

Assay Availability

- **Many of these assays are not commercially available**
  - Novascreen (cell free binding assays) no longer offered by Novascreen, but other vendors can provide
  - Odyssey Thera (protein complementation assays). Company is out of business, no known commercial source of these assays
  - Attagene (RNA-based transactivation assay) Company currently offers these assays
  - ACEA (Cell proliferation, real time impendence measurement) Company currently offers these assays
  - Tox21 (protein-based transactivation assays) These are produced by a US Government lab, and are not available as a service

- **However, variants of these assays could be developed by independent labs**
  - Specific aspects of assays may be patent protected, but basic technology is not

Proposed Path Forward

- **Identify laboratories to develop or offer variants of assays in the following classes**
  - Cell-free ER binding
  - Protein complementation / transcription factor dimerization
  - Transactivation
  - Cell proliferation
- **Use different cell types and readout technologies**
- **Validate each assay against the OECD in vitro reference chemical set**
- **Build the 4-assay subset model against the in vitro reference chemical set to determine assay weights**
- **Validate subset model against further chemicals shown to be ER agonist positive and negative from the current full model**
- **Use the new subset model to evaluate new chemicals**

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Acknowledgements

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  - Dr. Warren Casey, NICEATM
  - Dr. Maureen R. Gwinn, US EPA
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