



EPA ER / AR Minimal Assay Testing Plan

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EPA / JRC Call

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- EPA developed in vitro assay batteries / models to test for ER and AR activity
 - Intended to replace guideline ER in vitro and uterotrophic assays and AR in vitro assay
 - ER: Binding and TA (transactivation)
 - AR: TA
 - ER Pathway Model: 18 assays + mathematical model
 - AR Pathway Model: 11 or 12 assays + mathematical model
- EPA tentatively accepts ER and AR pathway models as “alternatives” to the guideline assays for the purpose of the EDSP (Endocrine Disruptor Screening Program)
- Models run for ~1800 chemicals
- Issue: These models (18, 11, 12 assays) are too complex to run for new chemicals

Moving to Minimal Assay Models

- We have demonstrated that it is possible to achieve close to the full model performance with fewer than the full set of assays
- Minimal ER assay model was subject to an OECD IATA in 2019
- It appears that moving to a Defined Approach will require that the individual assays be “validated”
- EPA is developing a plan to test a large number of pesticidal ingredients in a to-be-developed minimal assay battery
- Can JRC implement one or more assays to help with this validation effort?



Organisation for Economic Co-operation and Development

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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

- Binding assay (TG 493, commercial, Eurofins?)
- Proliferation assay (ACEA)
- Transactivation assay (Attagene trans)
- Transactivation assay (TG 455, run at EPA or commercially)
- Protein-protein interaction assay (PPI) (Simmons Lab EPA)
- QSAR model (CERAPP)(?)
- Cytotoxicity assays (at least simultaneous with TA and PPI)
- What is testing range? 0.01-100 μ M is typical for EPA, but guidelines may go higher



Candidate AR Assays

- Binding assay (OPPTS 890.1150, commercial, Eurofins?)
- Transactivation assay (Attagene trans)
- Transactivation assay, antagonist mode (TG 458, run at EPA or commercially), 2 different concentrations of the reference agonist
- Protein-protein interaction assay (PPI) (Simmons Lab EPA)
- QSAR model (CoMPARA)(?)
- Cytotoxicity assays (at least simultaneous with TA and PPI)
- What is testing range? 0.01-100 μ M is typical for EPA, but guidelines may go higher

- Full models used a mathematical model that produced a score (the AUC) for each of several modes
 - Agonist, antagonist, assay interference through a specific assay technology
- Drawbacks in use with minimal assay model
 - Some reviewers had difficulty understanding the models
 - This worked best when there were redundant assays for each technology
- Alternative simple arithmetic model
 - $\log(\text{potency}) = \text{mean}(\log(\text{potency}))$ across active assays
 - Calculate separately for each mode (agonist, antagonist)
 - If average potency < maximal testing concentration, the chemical is active



Validation Phase

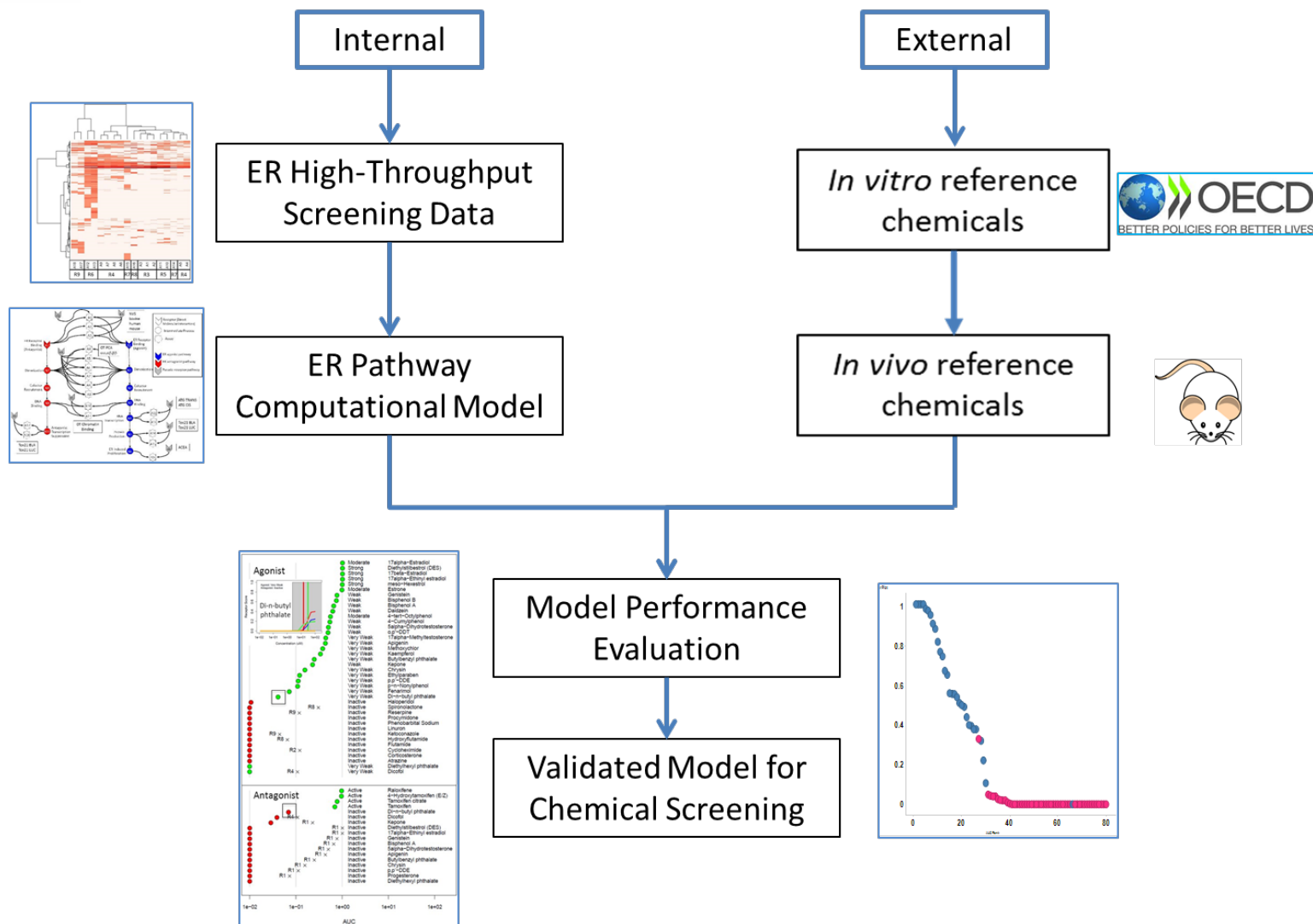
- Use 30-50 reference chemicals for each target
 - Range of potencies
 - Active and inactive
- EPA will source chemicals and have analytical QC performed
- JRC ask
 - Implement PPI ER And AR assays
 - Run with set of reference chemicals

- Select assays to run
- Select reference chemicals (maybe add assay interference chemicals)
- Develop validation approach that will be acceptable to OPP and other stakeholders
- Discuss making this an APCRA case study

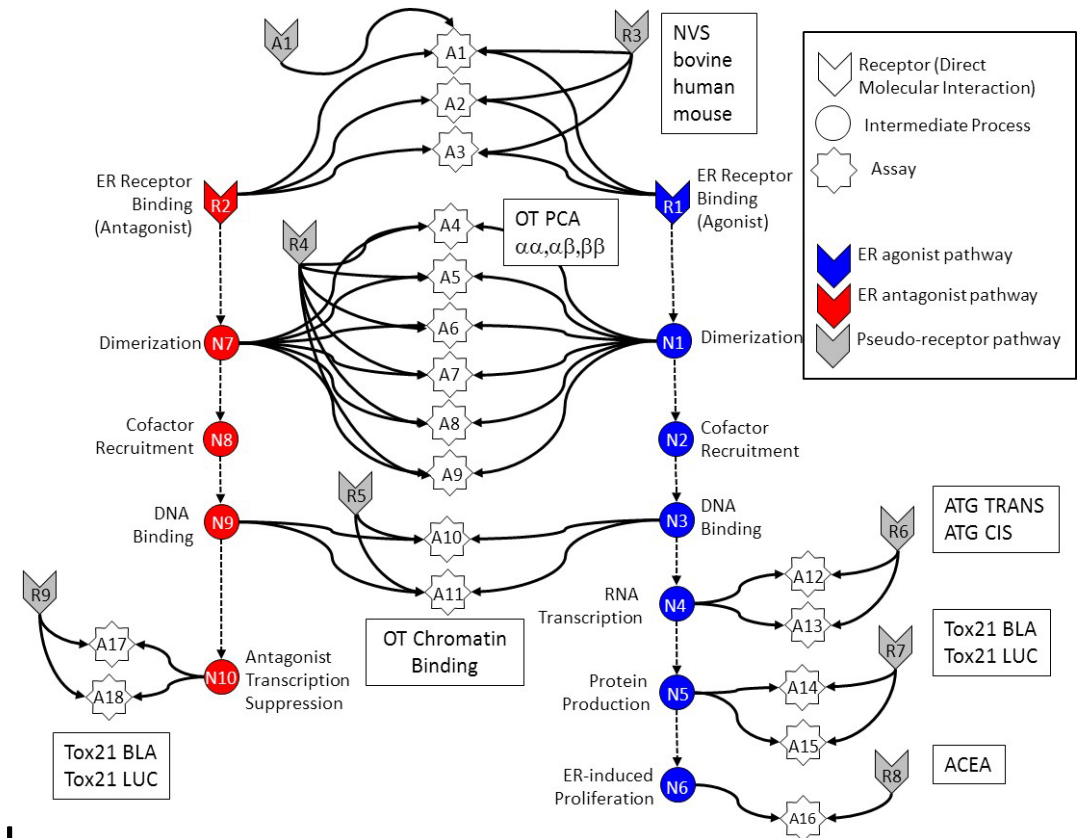


Background slides

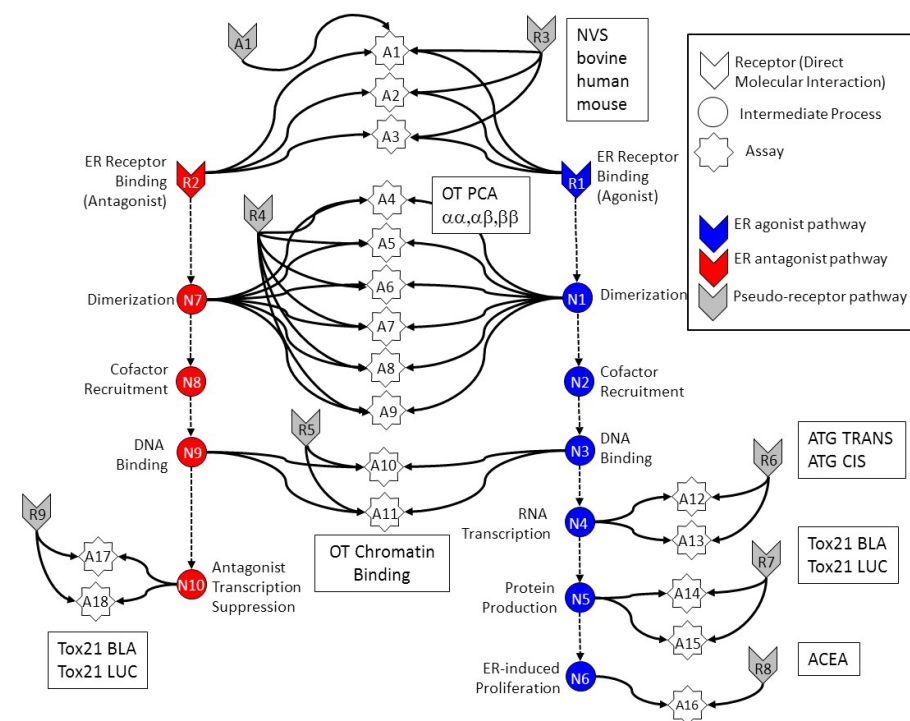
IATA Overall Approach

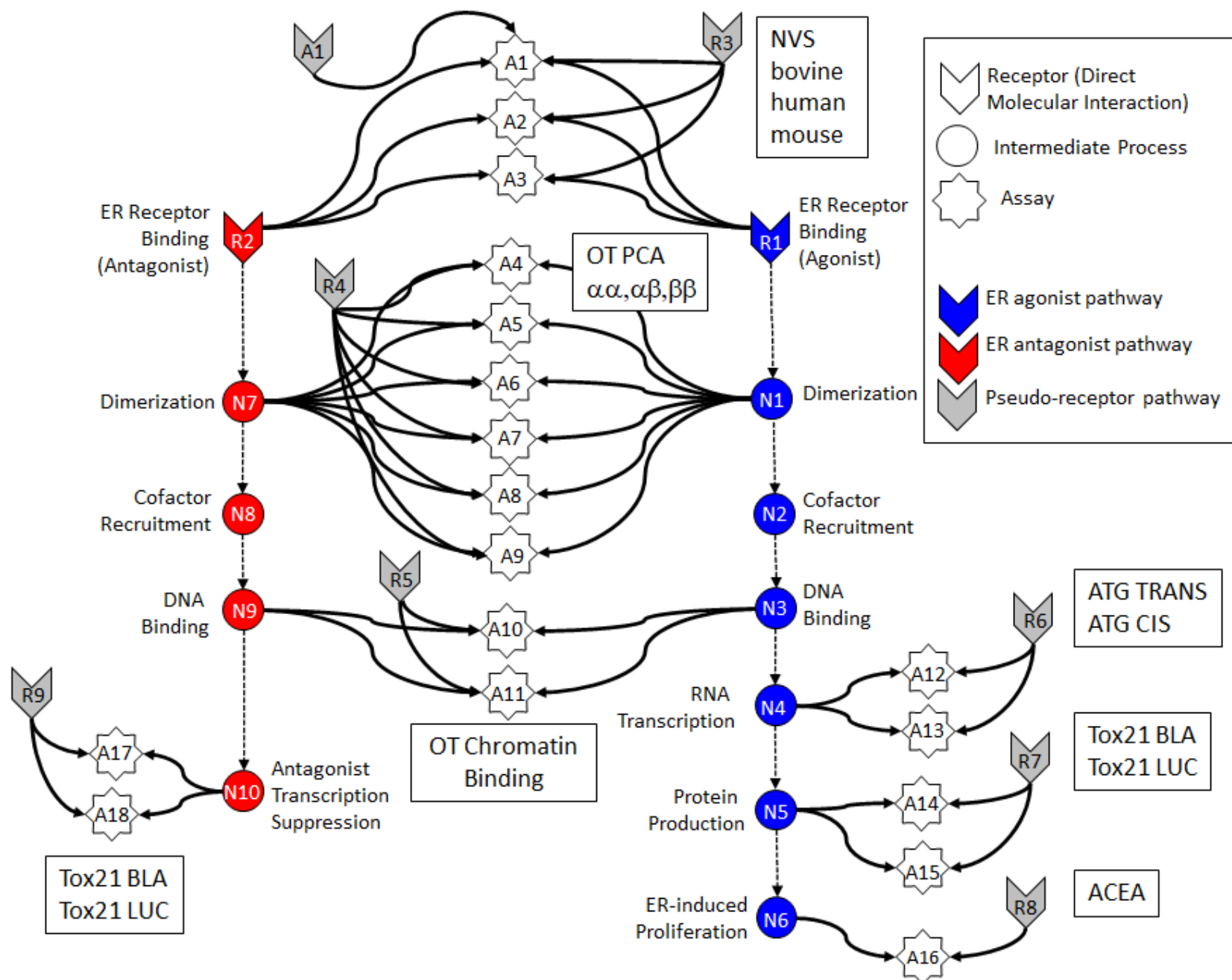


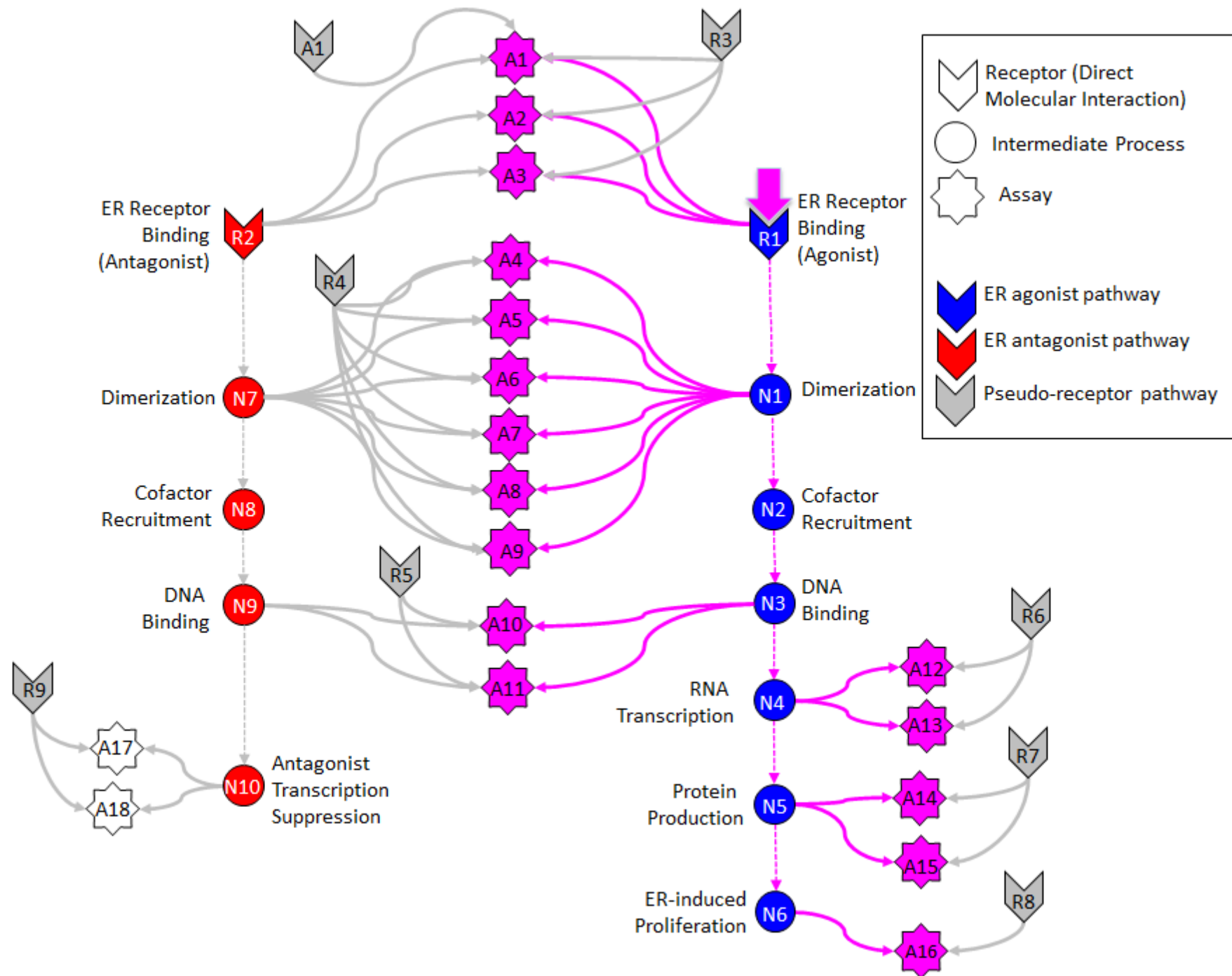
- Use multiple assays per pathway
 - Different technologies
 - Different points in pathway
- No assay is perfect
 - Assay Interference
 - Noise
- Use model to integrate assays
- Evaluate model against reference chemicals

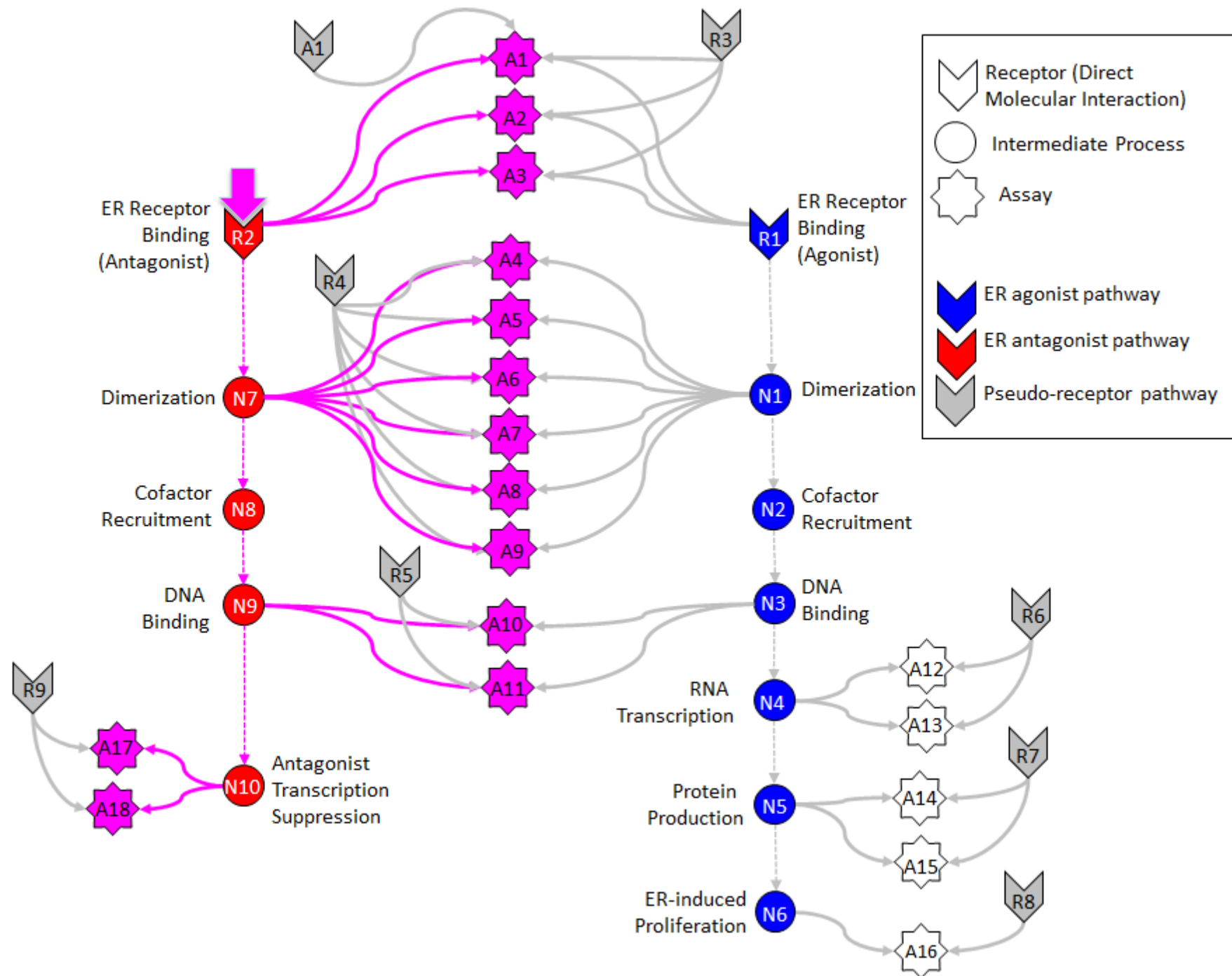


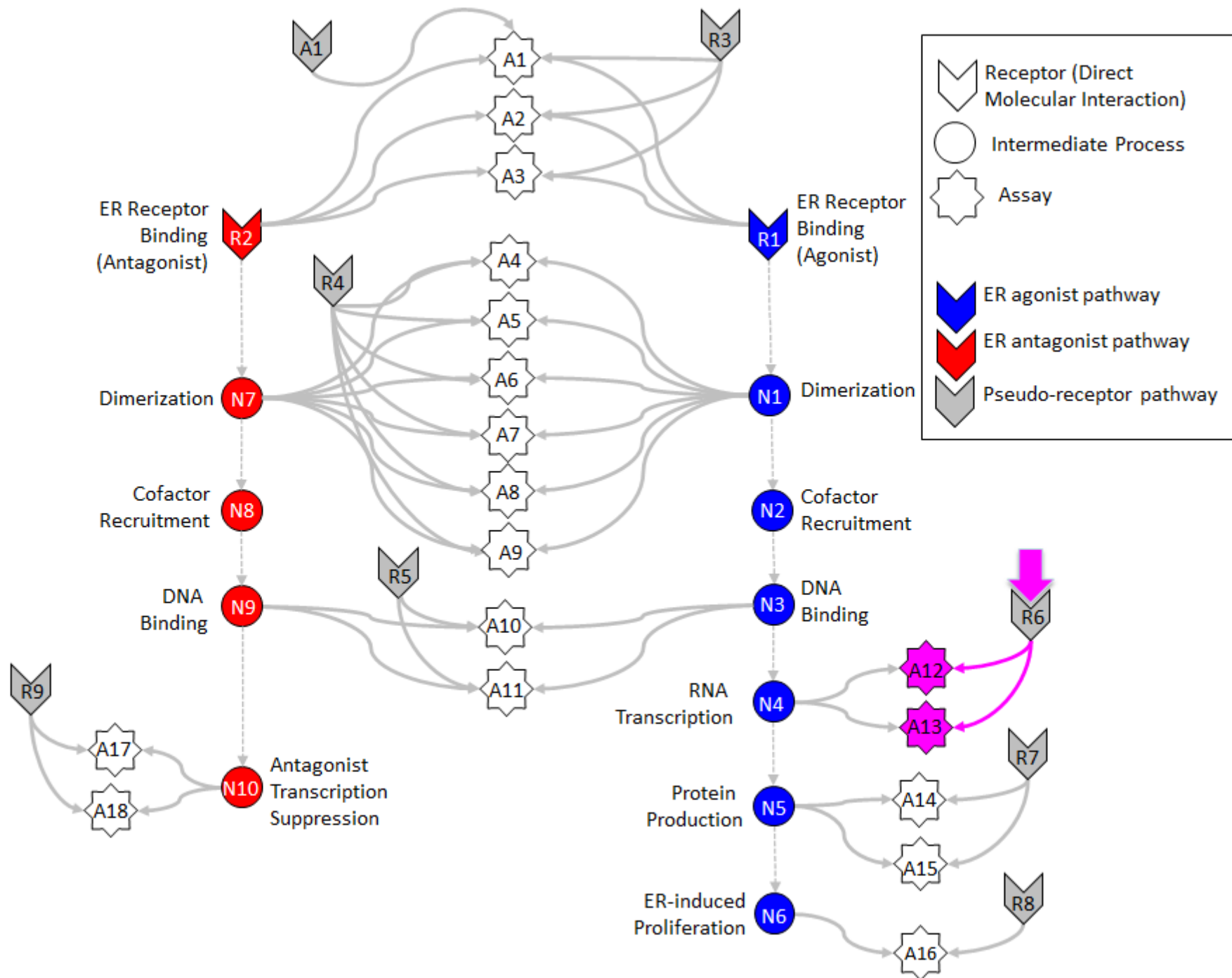
- For every concentration, look at the pattern of activity across the assays
 - If pattern is consistent with agonist activity, classify the chemical as an agonist
 - If pattern is consistent with antagonist activity, classify the chemical as an antagonist
 - Else, classify the chemical as acting through some technology or cell-type specific interference process





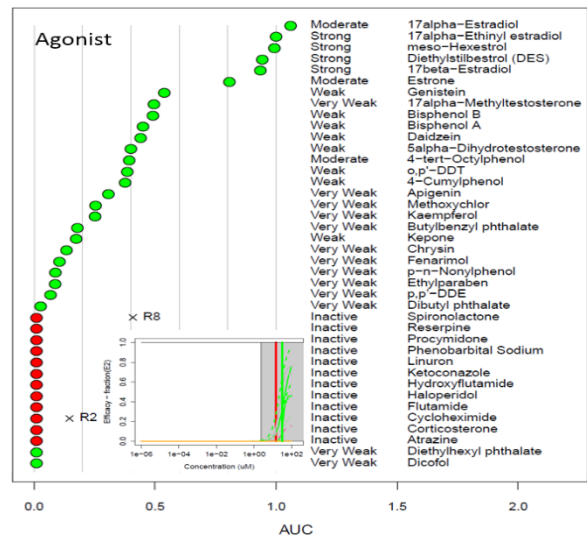






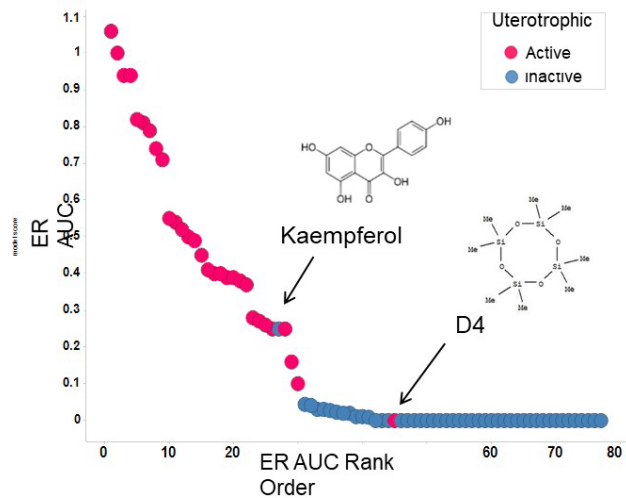


Demonstrate that the full model replicates reference chemical activity



True Positive	26 (25)
True Negative	11 (11)
False Positive	1 (0)
False Negative	2 (2)
Accuracy	0.93 (0.95)
Sensitivity	0.93 (0.93)
Specificity	0.92 (1.0)

In Vitro



True Positive	29
True Negative	46
False Positive	1
False Negative	1
Accuracy	0.97
Sensitivity	0.97
Specificity	0.97

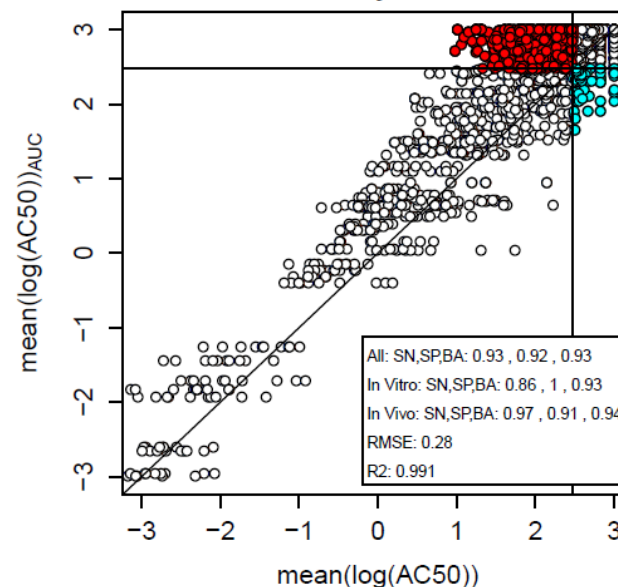
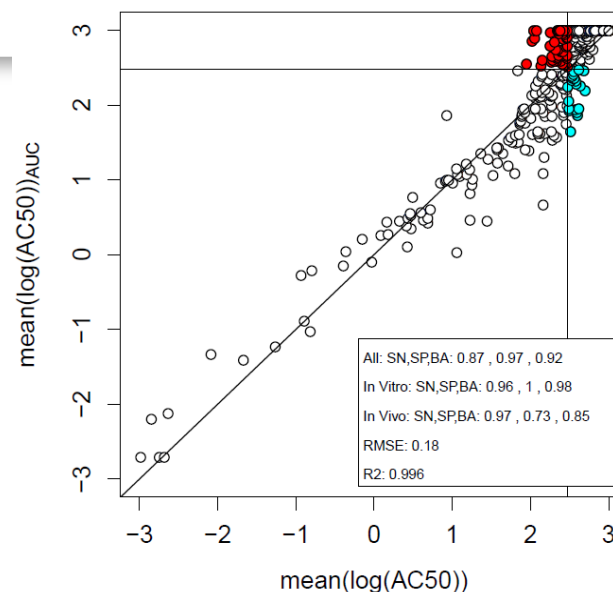
In Vivo

- Full model requires ...
 - 16 assays in agonist mode, many not commercially available
 - A complex mathematical model
 - But serves as benchmark (not the “truth”) for evaluating simpler models
- “Subset models” perform almost as well
 - Use a subset of as few as 4 assays (one can be a QSAR model)
 - Combining rule uses simple arithmetic (average potency across assays)
- The IATA and DA (defined approach) are built around these simple subset models



Demonstrating the performance of the simple model

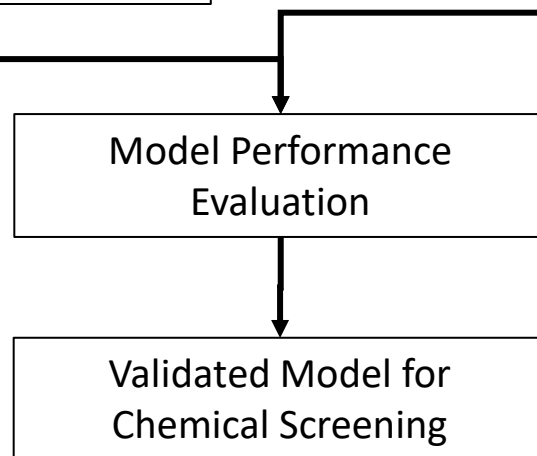
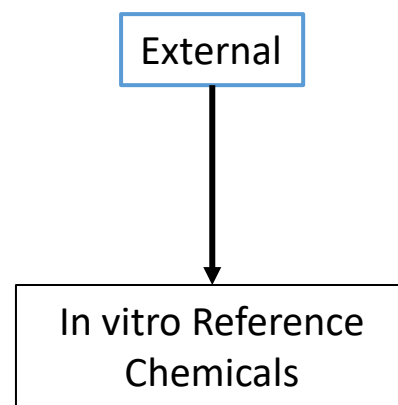
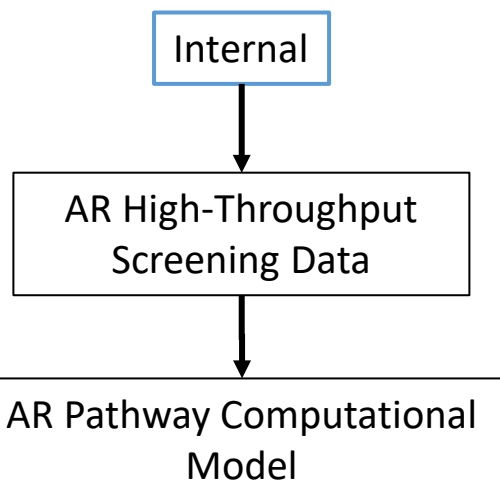
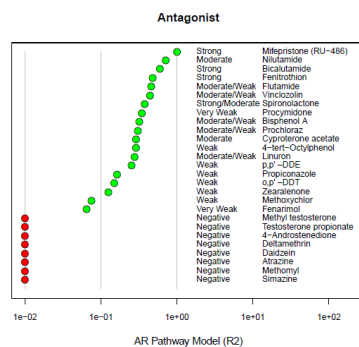
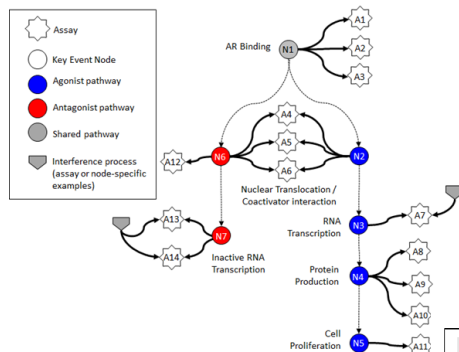
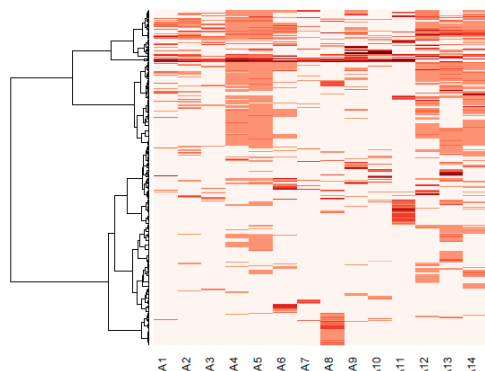
- Show that simple arithmetic can reproduce the mathematical model within the uncertainty of the model
 - Does not need to be perfect because current tests are variable
- Show that subsets (including QSAR model) are still accurate within the uncertainty of the model
- In both cases, chemicals that are misclassified are mostly “inactive” or “very weak”, and are ones that current tests may misclassify



Steps to getting OECD acceptance

- IATA was reviewed and published in 2018-2019
 - Demonstrates an approach of interest
 - 1-2 years
- Defined Approach is to be reviewed in 2020
 - Gives more details of implementation
 - Will include proposal for specific assays that could be generally available and process for “validation” of these
 - 1-2 years
- Test Guideline(s) will likely be needed
 - These would give more specific details on how to run each assay and combine the results
 - Multiple years
- However, EPA and EFSA are already using the full ER model in making regulatory decisions

Overall Approach



IATA Process

Run Assays

(Assays 1 - n,
cytotoxicity)

- Choose at least 5 in vitro HTS assays that fit the criteria as described. Briefly, the assays chosen must probe diverse points in the AR pathway and use diverse assay reporting technologies and cell types.
- The possible combinations of 5 assays that can be used together are listed in Appendix.

Import Data and Run Model

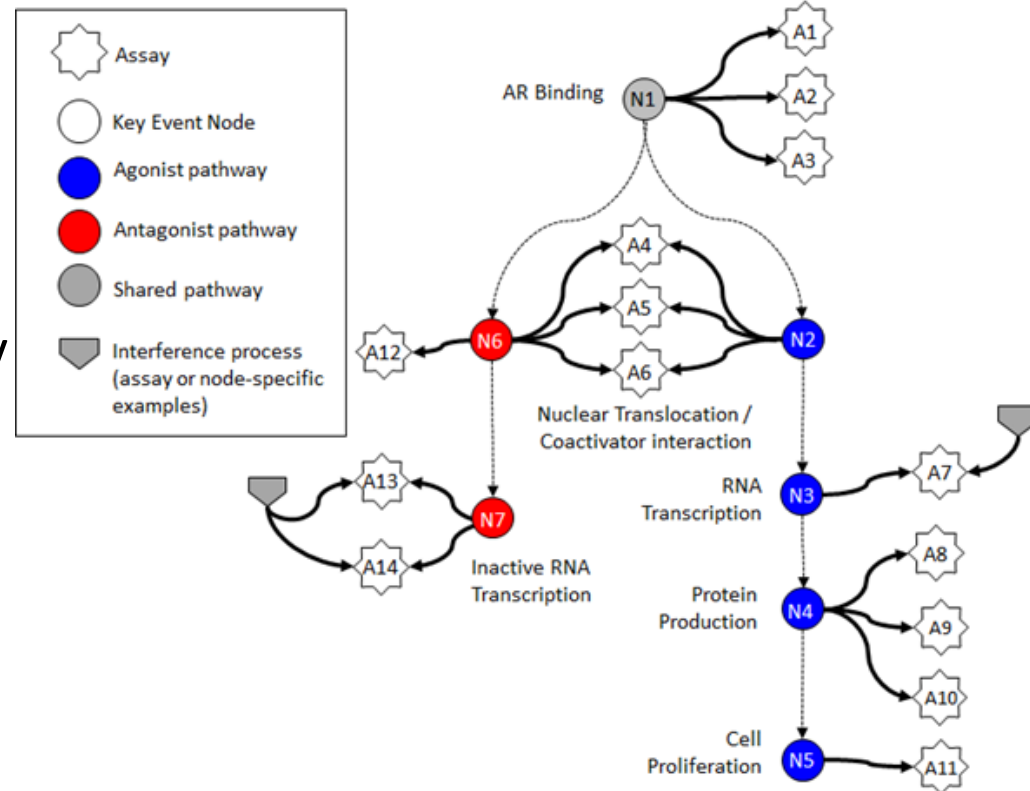
- The next step is to collect data from the assays into a format that the computational model's R-code can import and analyse. Ideally, this process will be performed automatically (electronically) to reduce the chance of user-input error.
- Run the biologically-based model and get an AUC value

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 concurrent cytotoxicity assays.
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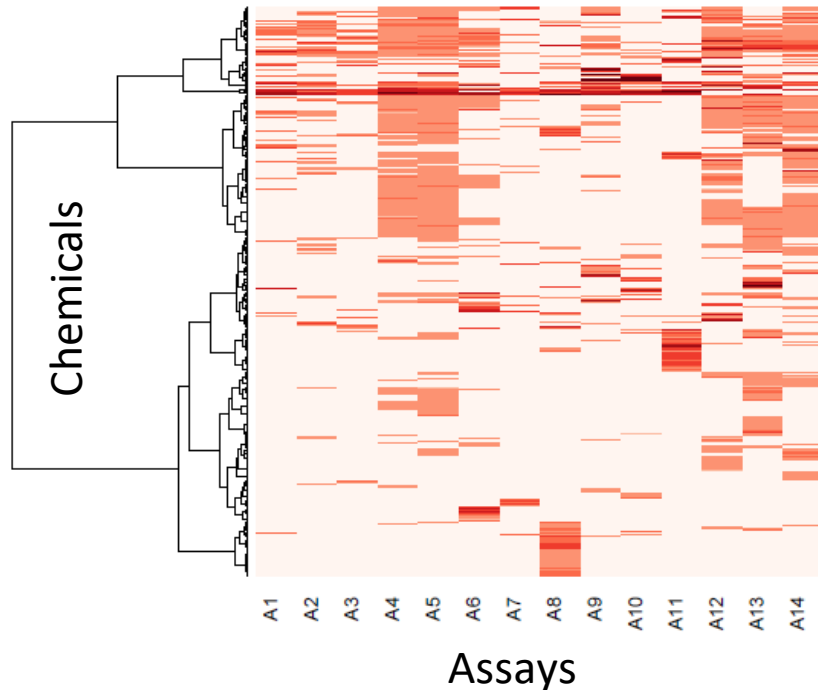
In Vitro Androgen Receptor Model

- 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



All In vitro assays have false positives and negatives

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

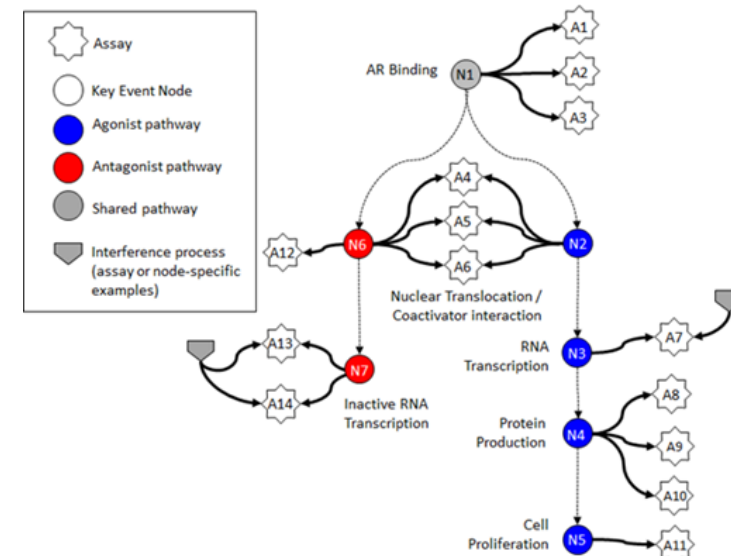


Much of this “noise” is reproducible

- “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



What Does the Model Do?

- For every concentration, look at the pattern of activity across the assays
 - If pattern is consistent with agonist activity, classify the chemical as an agonist
 - If pattern is consistent with antagonist activity, classify the chemical as an antagonist
 - Else, classify the chemical as acting through some technology or cell-type specific interference process



AR Subset Model

- Assume that the “full model” (9 antagonist assays) provides acceptable prediction of AR antagonist activity
 - Model based on detailed biology
 - Validated against in vitro reference chemicals that were developed for this model
- Build simple “subset models” using fewer assays
 - Validate against the full model over 1820 chemicals and in vitro reference chemicals
- Input to the model are assay-chemical concentration-response curves



Reference Chemicals

- No acceptable reference chemical set was available for AR activity
- Kleinstreuer et al. performed a systematic literature survey to identify potential reference chemicals
 - Chemicals were run in multiple labs in multiple assays of different cell types and readout technologies
 - Chemicals gave consistent results (positive and negative)
 - Positive chemicals spanned a range of potencies
 - Total of 28 antagonist mode reference chemicals identified
- Kleinstreuer et al: “Development and Validation of a Computational Model for Androgen Receptor Activity” (Chem Res Tox 2017)

IATA Process

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(Assays 1 - n,
cytotoxicity)

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Subset Model Input

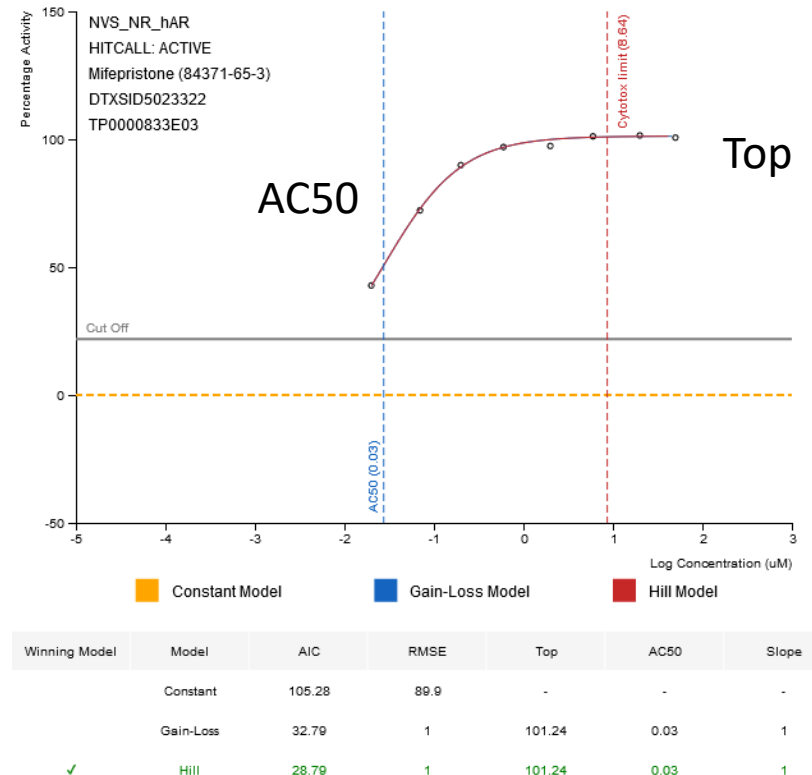
- Inputs to model are chemical-assay values: AC50 and Top
- Run assay in concentration-response mode
- Fit to model (e.g. Hill model)
- Calculate AC50 and Top

Example curve for Mifepristone

Data from:

EPA CompTox Chemicals Dashboard

<https://comptox.epa.gov>



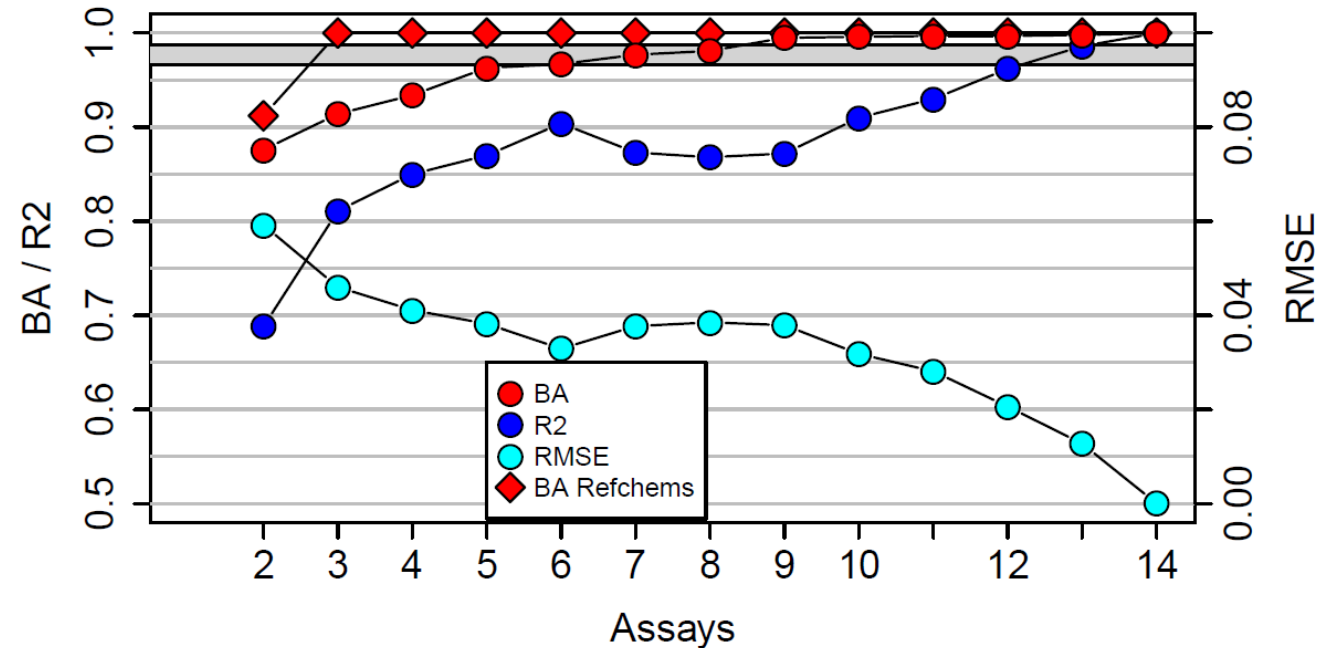


Building and Evaluating the Model

- For each set of assays (all combinations from 2 to 14), build the complete biologically-based AR model, including both agonist and antagonist activity
- Calculate several statistics on each subset pathway model
 - Sensitivity, specificity, balanced accuracy for all chemicals
 - Sensitivity, specificity, balanced accuracy for in vitro reference chemicals
 - Balanced accuracy is average of sensitivity and specificity
- Allows a user to select any model (i.e. any subset of assays) that provides high enough sensitivity, specificity, balanced accuracy

Statistical Results

Results for
Antagonist Mode



Balanced Accuracy, R^2 and RMSE for all chemical and BA for in vitro reference chemicals

Observe that with as few as 5 assays, there are subset models where reference chemical balanced accuracy is perfect and other subset models where all-chemical balanced accuracy is above 95%



Assay Availability Issue

- Many of these assays are not commercially available
 - Novascreen (cell free binding assays) no longer offered by Novascreen, but other vendors can provide equivalent assays
 - 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
 - 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 laboratories
 - 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 AR binding
 - Protein translocation / co-factor recruitment
 - Transactivation
- Use different cell types and readout technologies
- Validate each assay against the in vitro reference chemical set
- Validate subset model against further chemicals shown to be AR agonist positive and negative from the current full model
- Use the new subset model to evaluate new chemicals

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