A User's Guide for Accessing and Interpreting ToxCast™ Data

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February 13, 2017

This User's Guide, developed by Natalia Ryan, Ph.D. (Bayer Crop Science) in consultation with ACC's Computational Profiling Work Group, is intended to act as source of information for technical experts interested in accessing and evaluating EPA's ToxCast data and analyses. Although the information contained in this document is believed to be accurate, the content is for general information and use only; the authors and ACC disclaim liability for any inaccuracies or omissions that may have occurred. This document contains links to information that is publicly available on EPA's internet sites, and users should note that EPA's ToxCast data, data analysis procedures, interpretations of results and web site postings may be revised and updated at any time by the Agency.

If you find information in this User's Guide that is incorrect, misleading or incomplete, we would appreciate your comments and suggestions: please contact either Natalia Ryan Ph.D. (natalia.ryan@bayer.com) or Richard Becker Ph.D. (rick_becker@americanchemistry.com).

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Specific file names are indicated by *italicized font.*
Exact names or labels from the Dashboard or other website are indicated by **bold font.**

History and purpose of ToxCast and Tox21

ToxCast and Tox21 are high-throughput chemical screening programs designed in response to the 2007 NRC report "Toxicity Testing in the 21st Century: A Vision and a Strategy"[1], which called for the use of *in vitro* assays to determine the effects of thousands of largely untested chemicals on "toxicity pathways". The ToxCast (Toxicity ForeCaster) program is run by the National Center for Computational Toxicology (NCCT) within the EPA. The goals of this program are to identify *in vitro* assays and responses that are relevant to *in vivo* toxicity, to develop predictive models built on the results of multiple assays and chemical properties and to use these assays and models to screen environmental chemicals that have little or no available toxicity data and prioritize them for further testing.[2] The term Tox21 is often used to describe the concept of toxicity testing in the 21st century, but it is also the name of the formal collaboration established to address this issue of developing new toxicity testing strategies. Tox21 leverages the expertise of several federal agencies: US EPA (ToxCast), National Institutes of Health (NIH) National Center for Advancing Translational Sciences (NCATS, formerly the NCGC), National Institute of Environmental Health Sciences (NIEHS) National Toxicology Program (NTP) and the Food and Drug Administration (FDA).

Within the ToxCast program, chemical testing is coordinated by the NCCT, with data being collected by outside vendors and then analyzed and shared by the NCCT. At the completion of Phase II, ToxCast has screened nearly 2,000 chemicals in about 1080 assays. Chemical screening is also carried out by NCATS, collecting data for over 8,000 chemicals in more than 50 assays. This data is analyzed by the NTP and data is available in the Tox21 Toolbox[3]. The raw data was also provided to the NCCT to be analyzed through their data processing pipeline, so it is included in the ToxCast data sets with the assay designation "TOX21". For the remainder of this document, "ToxCast" will refer to the data acquired by both ToxCast and NCATS as analyzed by NCCT, containing a total of 1192 assay endpoints and 9076 chemicals.

ToxCast is an ongoing project, therefore versioning is quite important. The testing can be separated into phases and the phase indicates which chemicals and assays were tested. Chemicals and assays can be added or removed, and the data analysis methods are also evolving and being improved. Updating the data derived from a particular set of chemicals and assays results in a new "Data Release". Phase I and Phase II have been completed; the ToxCast program is currently in Phase III of testing. The Phase I data release was in January 2010. Phase II data was initially released in December of 2013, with updates in December 2014 and October 2015. A projected completion date for Phase III has not yet been announced. It is recommended to use the most up-to-date data available, and always be mindful of the data version used for publications when making comparisons.

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[1] Toxicity Testing in the 21st Century: A Vision and a Strategy. (2007) Committee on Toxicity Testing and Assessment of Environmental Agents, National Research Council, The National Academies Press. Washington, D.C. www.nap.edu.

[2] Judson RS, *et al*. (2010) In vitro screening of environmental chemicals for targeted testing prioritization: the ToxCast project. *Environ Health Perspect*. 118(4): 485-92.

[3] http://ntp.niehs.nih.gov/results/tox21/index.html

Data access

All the data generated by the ToxCast and Tox21 programs is made freely available to the public. The data can be accessed in three primary ways, depending on the needs and abilities of the user: the interactive online Dashboard, downloadable summary files or full data access through R scripts and a MySQL database. The most user-friendly way to access the data is through the iCSS ToxCast Dashboard[4] (**Figure 1**). The Dashboard allows for quick, easy access of data on single chemicals or assays. A more detailed explanation can be found below in the section "Using the ToxCast Dashboard".

Some download features are available through the Dashboard, however when large amounts of data are desired, users are directed to the ToxCast data download page[5]. The processing of multiple concentration screening data is complex, therefore the output contains several observations and statistical parameters including the hit call determination, AC10 and AC50. Summary files provide a snapshot of the data available at the time of the release and summary files for past releases are also available for download. From the data download page, select **Download ToxCast Summary Information** and open the zip folder titled *INVITRODB_V2_SUMMARY.zip*. The folder contains several files, each with a different parameter (hit call, winning model, AC50, top, bottom, etc.). Perhaps the most useful file is *oldstyle_ac50_Matrix_151020.csv*. This file combines the information provided in several other files to allow for easy comparison. All assays are listed across the columns and all chemicals are listed down the rows, identified by "code". The data is in the form of "NA" for not tested, "1000000" for not active and numerical values less than 1000000 indicate the AC50 in µM.

For the most advanced or specialized users, the full database can be accessed through MySQL and the use of R programming scripts. This allows the user access to all levels of data (not just the final processed results) as well as the ability to manipulate the data and even the processing methods. Instructions for downloading and using the MySQL database and the R package to manipulate the data are available from the data download page in the *ToxCast Data Pipeline Overview* document and will not be discussed further here.

Chemical information

Extensive information regarding chemical selection, procurement, management and applicability domain is available in a recent publication.[6] A full list of the chemicals included in ToxCast can be viewed on the Dashboard. This list can also be downloaded in two ways: 1) in the Dashboard, with **Chemicals** selected as the view (**Figure 1 A**), click the check mark (**Figure 1 B**); or 2) from the data download page, **Download ToxCast Summary Information** *-> INVITRODB_V2_SUMMARY.zip -> Chemical_Summary_151020.csv*.

[4] https://actor.epa.gov/dashboard/
[5] https://www.epa.gov/chemical-research/toxicity-forecaster-toxcasttm-data
[6] Richard AM, *et al*. (2016) ToxCast Chemical Landscape: Paving the Road to 21st Century Toxicology. *Chem Res Toxicol*. 29(8): 1225-51.

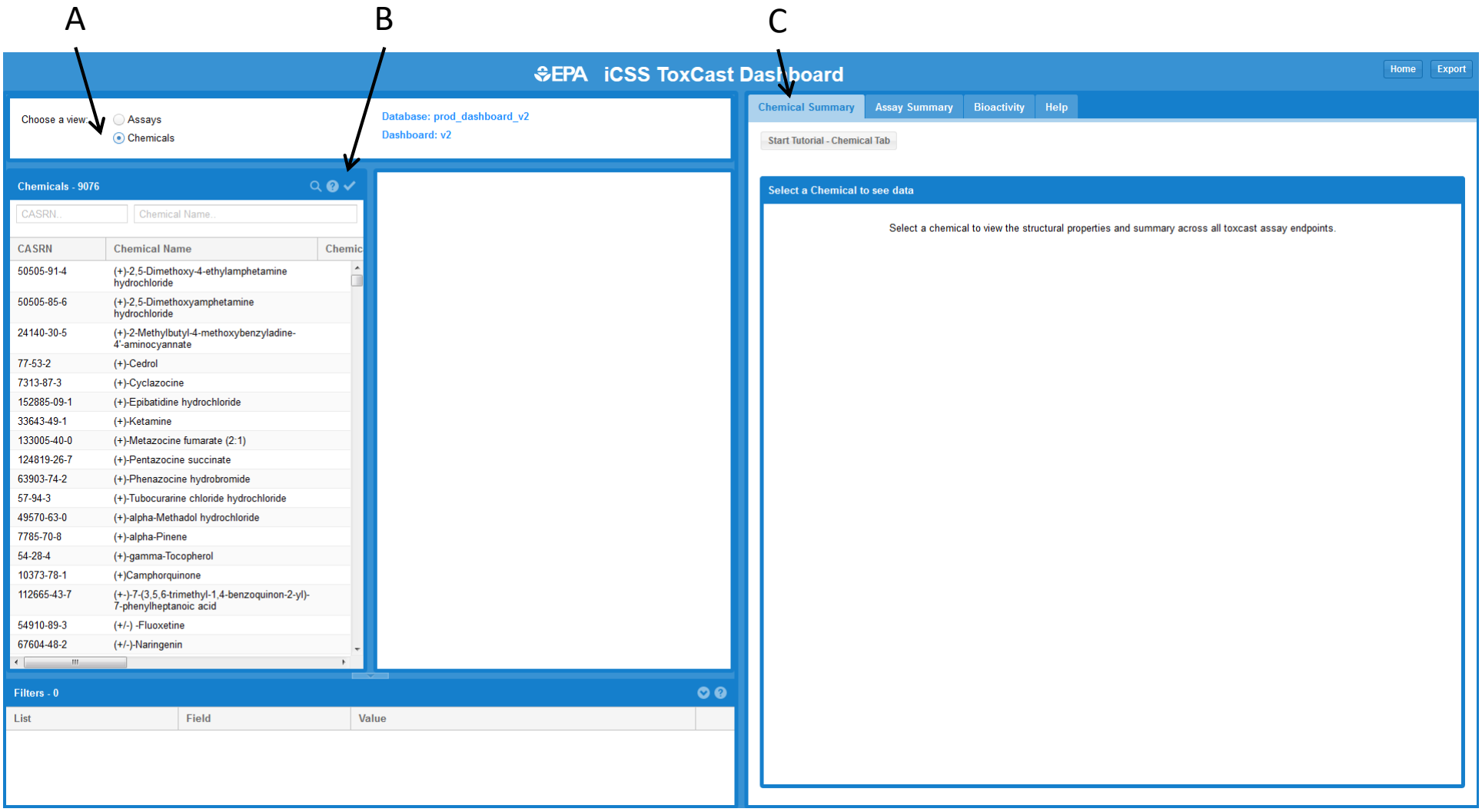

Figure 1. Screenshot of the ToxCast Dashboard. A – Select **Chemical** view. B – Click the check mark to download a .csv file of chemical information. C – **Chemical Summary** tab, select a chemical from the list on the left to display information.

Chemicals in ToxCast are identified by their CAS number and chemical name. ToxCast is connected to the DSSTox Database[7] so the **DSSTOX GSID** is listed on the Dashboard. In the data download files, chemicals may also be identified by a "code" which is simply the letter C followed by the CAS number with the hyphens removed (e.g. 50-28-2 = C50282) or by the "chid" which is a chemical ID assigned by ToxCast.

Analytical quality control is being performed for chemical samples plated for ToxCast to establish purity, identity, concentration and stability.[8] High-throughput LC-MS is used with follow-up GC-MS as necessary, and samples are analyzed at time 0 and again after 4 months at room temperature to assess sample stability. Quality control grades as well as the detailed results are available through the Tox21 Data Browser[9], and a link to this information is also provided in the **Chemical Summary** tab on the Dashboard (**Figure 1 C**).

Another way to access information about the chemicals included in ToxCast, along with thousands of other chemicals, is through the new CompTox Dashboard[10]. This resource provides chemical properties and identifiers as well as links to the ToxCast data and exposure information where available.

Assay information

Although the EPA's NCCT coordinates testing, provides the chemical samples and processes the data, the assays are mostly run by independent laboratories or vendors. The assay technologies are diverse and cover a wide variety of endpoints. A full list of assays utilized by ToxCast can be viewed on the Dashboard with **Assays** selected as the view (**Figure 2 A**). Expanded details are provided in the **Assay Summary** tab (**Figure 2 B**). The list with expanded details can be downloaded by clicking the check mark (**Figure 2 C**). Three spreadsheets with even more parameters are available from the data download page: **Download Assay Information** *-> Assay_Information_Oct_2015.zip.* The contents of the three files are described in the accompanying PDF file (*README_INVITRODB_V2_ASSAYINFO.pdf*). Most assays or the technology that they are based upon have been described in published literature. The PubMed URLs for these publications are found in the **Assay Summary** tab of the Dashboard (**Figure 2 B**), under the **Assay Citations** header as well as in the *Assay_Summary_151020.csv* file.

Assays are listed in the Dashboard by an abbreviated but informative annotation. The annotations are intended to provide: 1) assay identification information; 2) assay design information; 3) target information and 4) analysis information. For example, "CEETOX_H295R_ESTRADIOL_up" is from the vendor CEETOX, the assay is run in H295R (adrenocortical) cells, the hormone being measure is estradiol and the data was analyzed for activity in the "up" direction (looking to detect an increase in hormone level). Further explanations can be found in the following document from the data download page: *Assay Annotation User Guide*.

[7] https://www.epa.gov/chemical-research/distributed-structure-searchable-toxicity-dsstox-database
[8] https://www.epa.gov/sites/production/files/2015-08/documents/toxcast_chemicals_qa_qc_management_141204.pdf
[9] https://tripod.nih.gov/tox21
[10] https://www.epa.gov/chemical-research/chemistry-dashboard

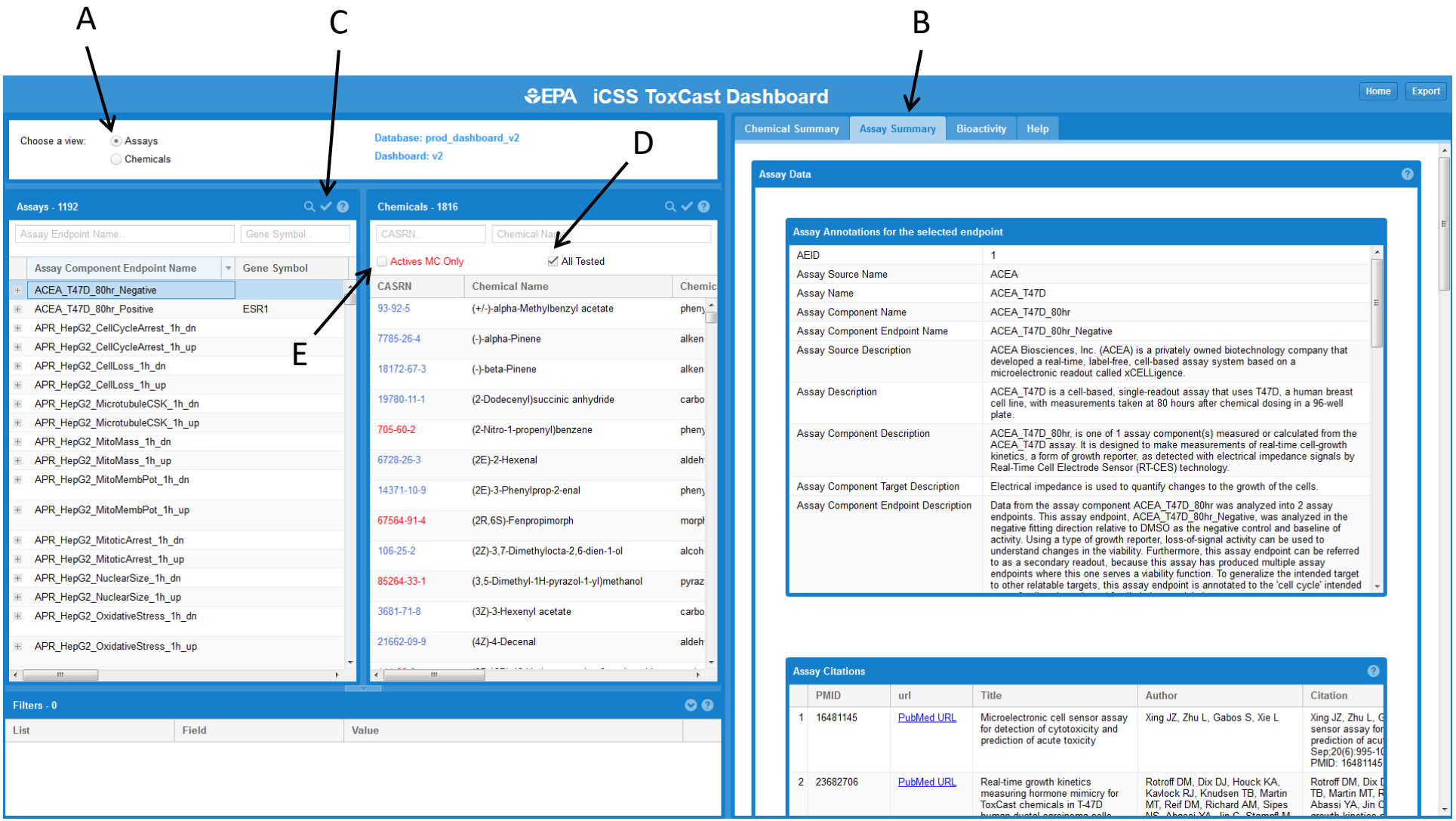

Figure 2. Screenshot of the ToxCast Dashboard. A – Select **Assay** view. B – Click the check mark to download a .csv file of assay information. C – **Assay Summary** tab, select an assay from the list on the left to display information. D – Uncheck this box to reveal all untested assays. E – Check this box to display only **Active** assays.

The ToxCast data analysis pipeline

The chemical screening data from ToxCast and Tox21 are processed and modeled using scripts written in the R programming language. The ToxCast program developed the *tcpl* package to process, normalize, model, qualify, flag, inspect and visualize the data (**Table 1**). In depth information can be found in two documents available from the data download page, *ToxCast Data Pipeline Overview* and *Download R Package -> tcpl.pdf*, so only a brief overview will be provided here.

	Description
Lvl 0	Pre-processing: Vendor/dataset-specific pre-processing to organize heterogeneous raw data to the uniform format for processing by the tcpl package
Lvl 1	Index: Define the replicate and concentration indices to facilitate all subsequent processing
Lvl 2	Transform: Apply assay component-specific transformations listed in the "mc2_acid" table to the raw data to define the corrected data
Lvl 3	Normalize: Apply assay endpoint-specific normalization listed in the "mc3_aeid" table to the corrected data to define response
Lvl 4	Fit: Model the concentration-response data utilizing three objective functions: (1) constant, (2) hill, and (3) gain-loss
Lvl 5	Model Selection/Activity Call: Select the winning model, define the response cutoff based on methods in the "mc5_aeid" table, and determine activity
Lvl 6	Flag: Flag potential false positive and false negative findings based on methods in the "mc6_aeid" table

Table 1. Adapted from *ToxCast Data Pipeline Overview* Table 4: Summary of the *tcpl* multiple-concentration pipeline

Raw data provided by a vendor or laboratory is processed, indexed, transformed and normalized using standardized methods (Lvl 0 – 3). Lvl 4 attempts to fit three chosen models to the concentration-response data, constant, Hill and gain-loss. The data may fit one or more of the models, or none at all. In Lvl 5, if any models were sufficiently fit, this chemical-assay pair is considered "active" (hit call = active). Then the winning model is chosen based on the lowest AIC (Akaike information criteria) value, essentially indicating the model that best fits the data. The response cutoff, AC50 (concentration where half maximal activity occurs) and other parameters are calculated based on the winning model. For chemical-assay pairs where no models were sufficiently fit, the hit call is "inactive" and a winning model is not chosen. The final step is to assign "flags" or warnings to the data when methods in the data processing pipeline have identified possible false positive or false negative findings. Flags will be discussed in more detail in a later section.

In addition to the hit call, AC50 and other numerical parameters resulting from the data analysis, dose-response curves are available to visualize the data. Dose-response curves can be accessed from the Dashboard by selecting a chemical and assay pair (one example highlighted in blue in **Figure 3**). Then select the **Bioactivity** tab (**Figure 3 A**) and click the icon with the black arrow (**Figure 3 B**). The dose-

response curve will appear in a new window (**Figure 4**, top panel) with the AC50 indicated by a blue line on the graph and in the table to the right. All three models are shown in the table, and the winning model is indicated in bold. Outside of the Dashboard, a file of all dose-response curves is available for download, but the file size is 15.8 GB and use would be limited. Curves can also be accessed through the MySQL database. This allows the user to create customized files with all the plots for a single chemical, a single assay or any other unique combination. The visual output is slightly different than from the Dashboard (**Figure 4**, bottom panel), but the same information was used to generate the curves. The curves downloaded from the MySQL database also have some of the numerical parameters displayed on the right-hand side of the page. The text for the winning model will be colored red (HILL in **Figure 4**). The AC50 is in the "val" row and the "ga" column for the winning model, 0.906 for the example in **Figure 4**. The AC50 is given here in log10 micromolar, so the value in micromolar is 8.05. This corresponds to the vertical line on the graph, and also with the AC50 given on the curve accessed from the Dashboard (slightly different likely due to rounding).

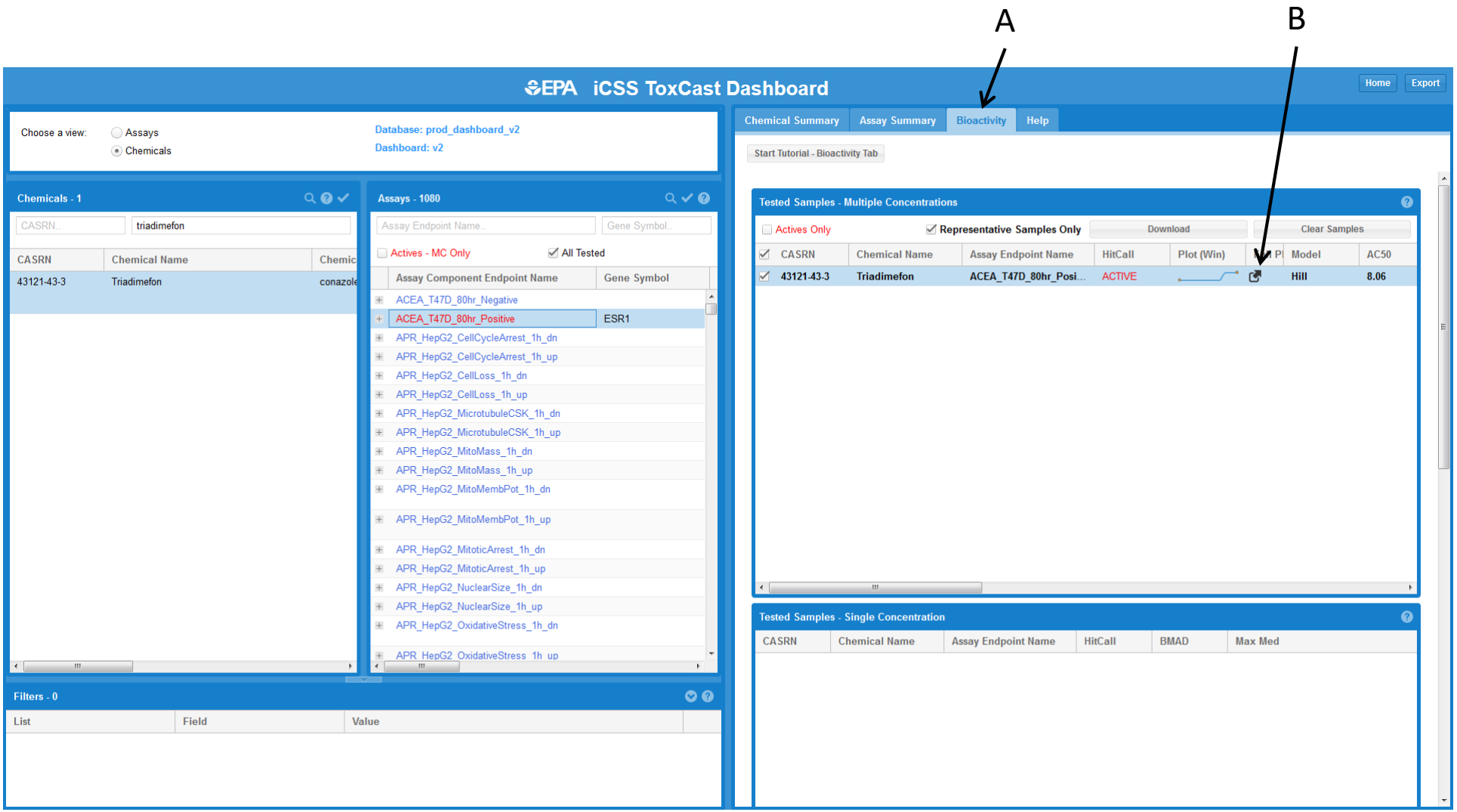

Figure 3. Screenshot of the ToxCast Dashboard with a chemical and assay pair selected (highlighted in blue). A – Select the **Bioactivity** tab to reveal HitCall, AC50, AC10, etc. B – Click the icon with the black arrow to open the dose-response curve in a new window.

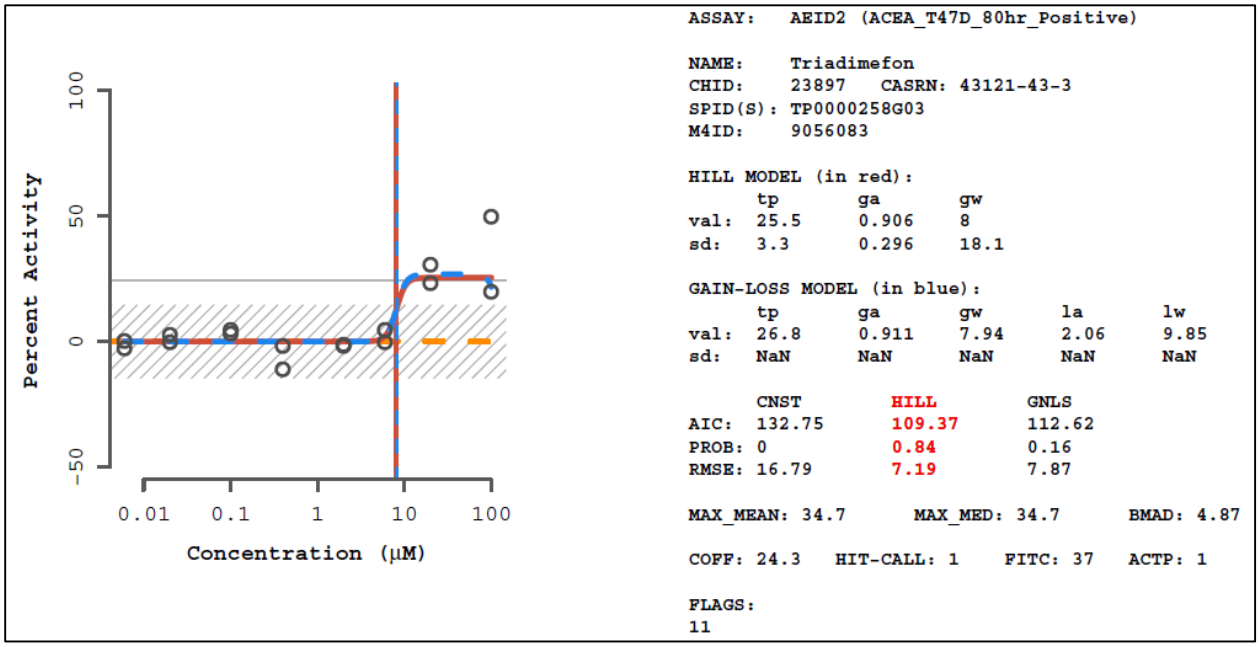

Figure 4. Example dose-response curves. Top – Screenshot from the ToxCast Dashboard. Bottom – Downloaded from the MySQL database.

Using the ToxCast Dashboard

The iCSS ToxCast Dashboard is an interactive tool to facilitate visualization and use of the chemical screening data from the ToxCast and Tox21 projects. Tutorials are available on the Dashboard that provide "click-by-click" instructions and descriptions of most of the Dashboard features. To access the primary tutorial, select the **Help** tab (**Figure 5 A**), then expand the section titled **How to Use the iCSS Dashboard** and click **Use Case Tutorial**. The **Help** tab also contains a **Glossary** with definitions to terms and abbreviations used in ToxCast and a list of **Frequently Asked Questions**. There are additional tutorials in the top left corner of the **Chemical Summary** (**Figure 5 B**) and **Bioactivity** (**Figure 5 C**) tabs. Hovering your mouse over most icons on the Dashboard will reveal descriptive text (**Figure 5 D**).

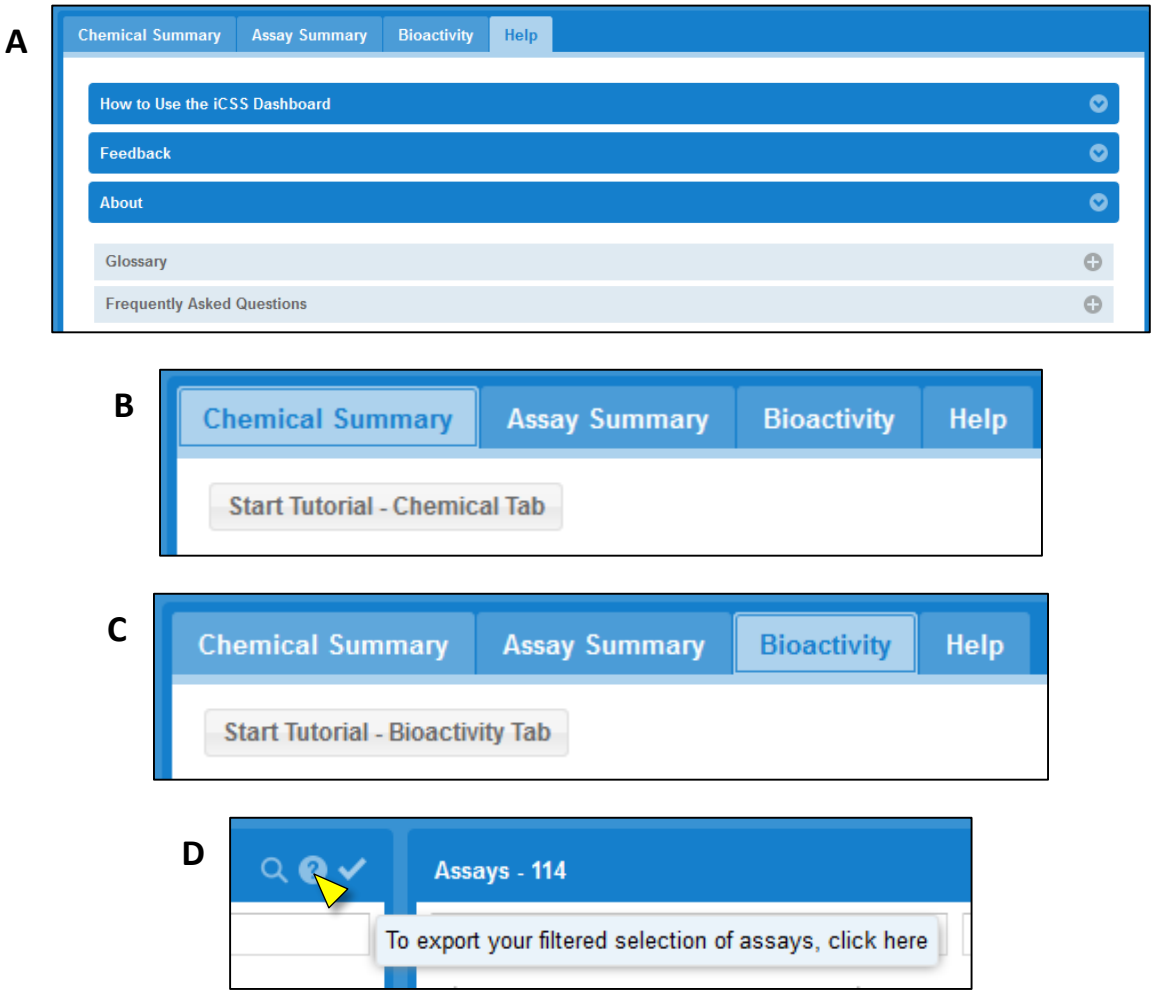

Figure 5. Screenshots of various help features on the ToxCast Dashboard. A – **Help** tab with a tutorial, glossary and FAQ. B – Tutorial for Chemical features. C – Tutorial for Bioactivity features. D – Hover over any icon for descriptive text.

Data in the Dashboard can be viewed in two different ways. First, you can select an assay and view the data for all chemicals tested in that assay (**Figure 2 A**). Alternatively, you can select a chemical and view the data for all of the assays tested for that chemical (**Figure 1 A**). After selecting either **Assays** or **Chemicals**, you can scroll to browse through the list, or begin typing an **Assay Endpoint Name**, a **Gene Symbol**, a **CASRN** number or a **Chemical Name**. The search functions will find the text you enter anywhere in the entry. For example, typing "50" in the **CASRN** box will bring up 50505-91-4, 1502-95-0, 25899-50-7, etc. and you can refine the list by continuing to enter more of the CAS number you are searching for.

Once a chemical has been selected (by clicking), all the tested assays are automatically displayed. To see the full list of 1192 assay endpoints, uncheck the box for **All Tested** (**Figure 2 D**); assays that were not tested for that chemical will appear in gray. Within the list of tested assays, red text indicates Active, blue text indicates Inactive – tested at multiple concentrations, black text indicates Inactive – tested at a single concentration. Dose response curves are not available for assays tested at a single concentration; information on the samples tested is given only in the **Tested Samples – Single Concentration** section of the **Bioactivity** tab. To display only the active assays, click the box for **Actives – MC Only** (**Figure 2 E**).

The **Chemical Summary** tab (**Figure 1 C**) provides several pieces of information. Chemical structure and identifiers including SMILES, InChI and InChI Key are presented. The quality control information as provided by Tox21 is summarized and links to the full reports are given. A list of all the samples of the chemical in ToxCast, PhysChem properties, CPCAT Categories and Exposure Predictions are provided when available. Two visualizations of the full data set for each chemical are also shown. One plots all active endpoints by AC50 and top of the curve; you can deselect groups of assays by clicking them in the list on the right of the graph. The other visualization is a histogram of the active endpoints grouped by intended target family. Data for the groups can be displayed in a table format by clicking one of the bars in the histogram.

The **Assay Summary** tab provides a variety of information about the selected assay. Annotations, parameters and descriptions about the assay technology and conditions are listed. Links to PubMed for any related publications are given. The data processing methods that were applied in the different levels of analysis for this particular assay are listed and described. A table shows the reagents and conditions used both for the cell culture (if applicable) and the assay for bioactivity. There are links to any Adverse Outcome Pathways (AOPs) that may exist for the intended target of the assay along with **Assay Tags** you can click to use as filters for the assay list. Graphs are used to present the summary statistics for all chemicals that were active in the chosen assay (binned by AC50 or scaled top value) and the numerical data is also provided.

The **Bioactivity** tab provides the bulk of the actual data for a selected chemical-assay pair. When a chemical and assay have been selected from the lists on the left-hand side of the page, the assay endpoint will appear in the top panel of the **Bioactivity** tab. A default collection of columns containing descriptive parameters are shown, but more parameters are available, and this can be customized by clicking the arrow next to any column title, selecting **Columns** and then choosing the desired parameters to display (**Figure 6**). The data for any chemical-assay pairs currently shown in the table can be exported to a .csv file by clicking the **Download** button, however a standard set of parameters will be exported regardless

of which columns are currently displayed in the table. In this downloaded file, first determine which model is displayed in the **WinningModel** column. The AC50 is either in the **HillGa** column if the hill model was chosen, or **GnlsGa** if the gain-loss model was chosen, and the value is in log10 micromolar. Dose-response curves can also be accessed through the **Bioactivity** tab, as described above (**Figure 3 B**).

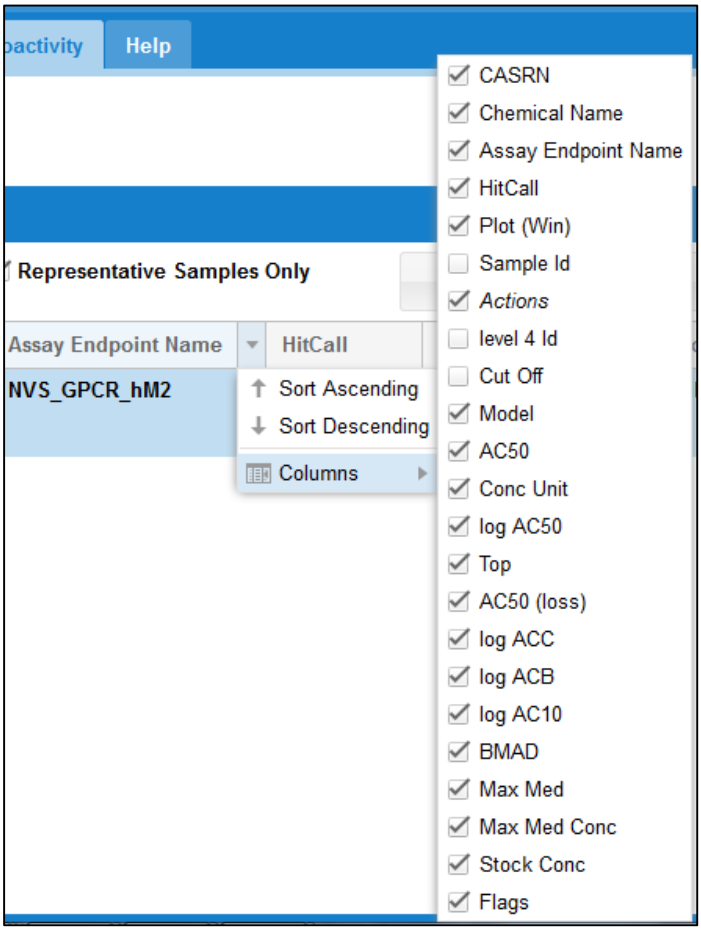

Figure 6. Screenshot showing how to add or remove parameters from the results table in the **Bioactivity** tab.

Interpretation of ToxCast results for a single chemical

As mentioned in the Introduction, ToxCast was created as a screening program. Assays were designed to maximize throughput, data processing was designed to minimize false negatives, and the data was intended to be used altogether for computational exercises and modeling to identify patterns. However, the question is now often being asked "What does ToxCast say about chemical X?". The availability of data permits users to answer this question, but the appropriate utilization of ToxCast data in this context requires deeper analysis and consideration of some key factors. The NCCT acknowledges this with a word of caution to users on the opening screen of the Dashboard:

- The activity of a chemical in a specific assay does not necessarily mean that it will cause toxicity or an adverse health outcome. There are many factors that determine whether a chemical will cause a specific adverse health outcome. Careful review is required to determine the use of the data in a particular decision context.
- Interpretation of ToxCast data is expected to change over time as both the science and analytical methods improve.

The meaning of "careful review" of the data depends on the use case, and the factors presented below are important when determining the *in vitro* bioactivity or potential modes of action predicted by ToxCast for a single chemical. This is not likely an exhaustive list, but provides a good basis to start an analysis. Many points are supported by examples, and data for the fungicide triadimefon has been used to illustrate features or issues of data interpretation.

Creating a list of active assays

The ToxCast Dashboard contains many useful features for quickly determining the number of active assays for a particular chemical, for checking the activity in a particular assay, for looking at summary statistics for an assay or a chemical and for viewing dose-response curves. However, the ability to download the list of active assays for a particular chemical and their AC50s is quite cumbersome through the Dashboard. One could select all the active assays one at a time, move them to the Bioactivity tab, export the .csv file and then go through the steps mentioned above to identify the AC50. Alternatively, after selecting a single chemical, clicking the Export button in the top right-hand corner of the Dashboard will produce a very detailed list of the results for that chemical. The list could be filtered for active hits (**hitc** = 1) and then the **modlGa** is the AC50 for the winning model. The main challenge with using this file is that the assay names are not given. The m4id must be used to cross-reference and look up the assay that goes with the provided data. There is a file of m4ids (*m4id_Matrix_151020.csv*) in the *INVITRODB_V2_SUMMARY.zip* file mentioned in the **Data Access** section of this document, however the format of the table does not allow for easy searching and matching.

The most convenient way to obtain a list of the results for a single chemical is through the file *oldstyle_ac50_Matrix_151020.csv* found in the *INVITRODB_V2_SUMMARY.zip* file. The CAS number for a chemical should be converted to the code (the letter C followed by the CAS number with hyphens removed, e.g. C43121433) and then use the Find feature in Excel (Ctrl+F) to search for this code. Copy the row containing the desired code, along with the title row, and paste transposed into a new spreadsheet so that there is a column of assays followed by the results for the desired chemical. The list can be sorted by AC50 to group together the active assays (those with a numerical value for the AC50), the inactive assays (AC50 = 1000000) and assays that were not tested (NA). Differentiating between not tested and not active can be important when an expected positive assay for a given chemical is not a hit. Once the list of active assays has been generated, each individual assay result can be analyzed for the following features listed below.

Curve/data quality

Due to the high-throughput nature of the ToxCast screening program and the large amounts of data collected in the program, automated data processing is absolutely necessary. Also, for a basic user of the data, it is often accessed only as a hit call (active or inactive) or as a hit call with the AC50. Simplifying the data to this level may be necessary for some computational exercises, but when considering the data for a single chemical, a more in depth analysis of the data used to derive this hit call and AC50 is appropriate. Users should consider both the validity of the hit call and the plausibility of the AC50. The following paragraphs will present suggestions for a qualitative analysis of these parameters. More sophisticated, quantitative methods for improving or refining the hit call and AC50 determination processes are definitely possible, but are not the focus of this document.

Not all responses that the data processing pipeline labels as "active" appear to be truly positive results. The ToxCast *tcpl* data processing package was created for screening and was therefore designed to minimize false negatives. False positives likely exist for most chemicals, and the end user is responsible for identifying these during their analysis. The first place to look to address the question of hit call accuracy is the dose-response curves provided in the Dashboard. There are many examples that could be shown to illustrate possible false positive hits, but only two are shown here. In **Figure 7**, the curve for BSK_3C_Proliferation_up fits the gain-loss model, but the single point above baseline is barely over the threshold for activity. The curve for NVS_GPCR_hM4 also has activity barely over the threshold, and the response does not consistently increase with increasing concentration of chemical.

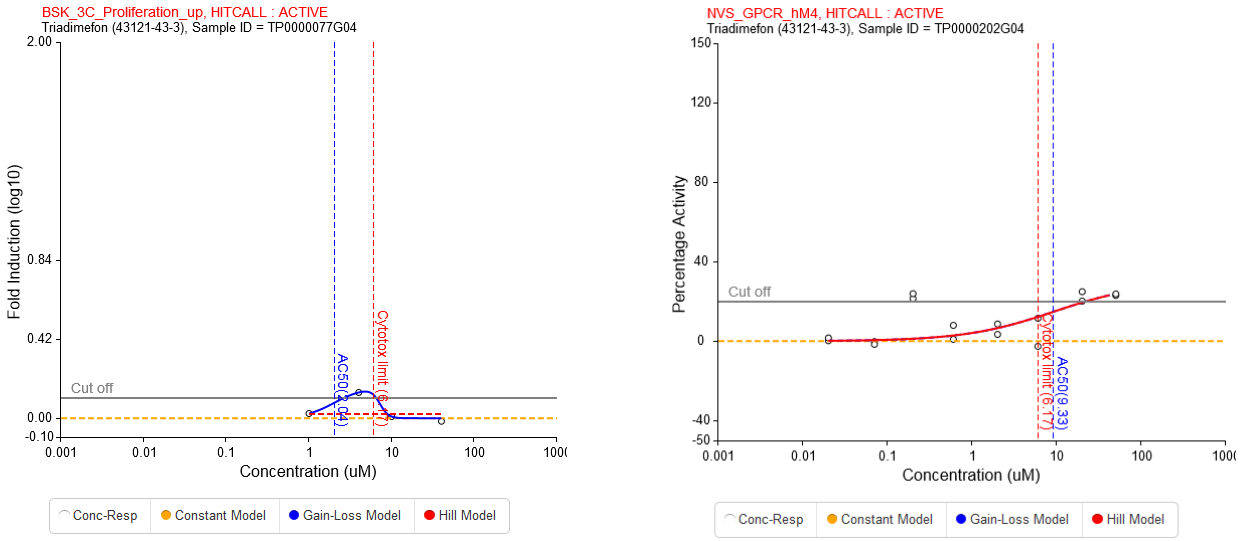

Figure 7. Example dose-response curves showing possible false positive results.

The ToxCast program has acknowledged that false positive and negative hit calls are possible using the automated methods, and have thus added a processing step (Level 6) to assign "flags" or warnings to the data. By definition, "Level 6 processing uses various methods to identify concentration series with etiologies that may suggest false positive/false negative results or explain apparent anomalies in the

data."[11] The possible flags assigned by ToxCast are listed in **Table 2**. While the flags are a helpful addition to the data analysis process, their assignment is also completely automated, and thus prone to some error. Careful examination of dose-response curves shows that within a group of curves receiving a certain flag, there is still a wide range of responses. Some curves with flag 10 for example may appear plausible, but others appear to be clear false positives. Therefore, it may not be the best practice to set hard filters based on flags, but flags are definitely an important consideration when analyzing a list of positive results.

Flag	Description
6	Only highest conc above baseline, active
7	Only one conc above baseline, active
8	Multiple points above baseline, inactive
10	Noisy data
11	Borderline active
12	Borderline inactive
15	Gain AC50 < lowest conc & loss AC50 < mean conc
16	Hit-call potentially confounded by overfitting
17	Biochemical assay with < 50% efficacy

Table 2. The flag labels and descriptions used by the ToxCast analysis pipeline to describe the data and hit calls.

Flags can be found in several places. In the Dashboard, flag descriptions are listed in one of the columns in the data table in the **Bioactivity** tab, but the flag label or number is not given here (**Figure 8**, top). When dose-response curves are displayed from the Dashboard, there is a place for listing flags, but currently, the flag information is not carried over to this location. All curves show "**none**" in the space designated for flags (**Figure 8**, bottom). This is an important note for users who are only accessing data through this curve display page. A full listing of flags can be found in one of the downloadable Summary files, *AllResults_flags_151020.csv*. Not all curves will have flags assigned to them, the "best" responses will have no flags, and some curves will have multiple flags.

[11] *ToxCast Data Pipeline Overview* (https://www.epa.gov/chemical-research/toxicity-forecaster-toxcasttm-data)

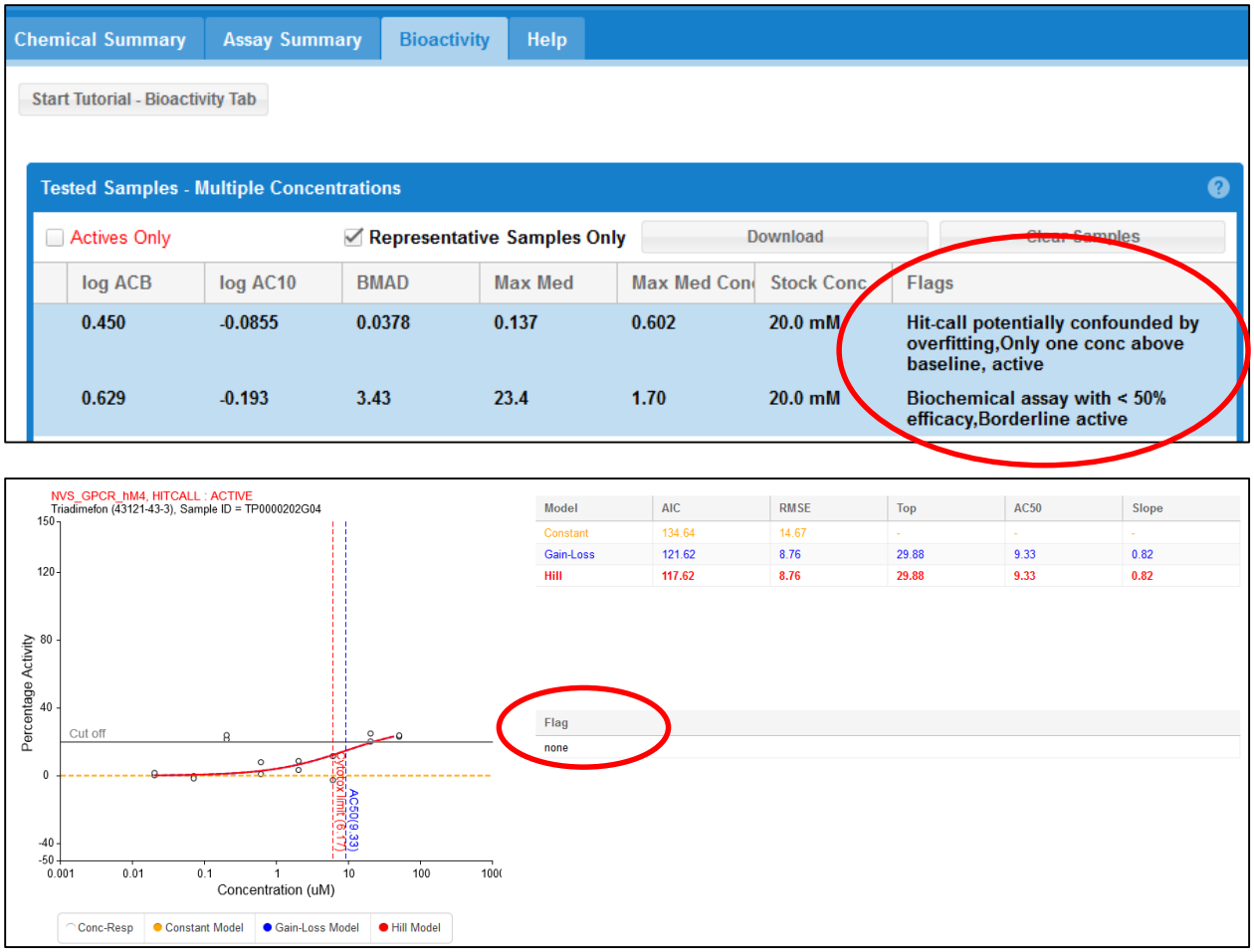

Figure 8. Two locations of Flags on the ToxCast Dashboard. Top – In the table of the **Bioactivity** tab. Bottom – In the dose-response curve view.

After determining the accuracy of the hit call, it is also important to consider the AC50 that has been calculated for the response. Errors of this nature seem to be less frequent than false positive hit calls, but they still exist and should not be overlooked. An example is provided in **Figure 9**, top panel. The AC50 for this assay is given as 0.000365 µM, which at first glance suggests that the chemical is very potent in this assay. A closer look at the data reveals that the lowest concentration tested was 0.006 µM, and that was the only concentration that produced a response. This may not be the ideal example since this curve would likely be labeled as a false positive due to the shape of the curve, but it does illustrate the point that the calculated AC50s may not accurately reflect the data. Another example that appears to be a more plausible hit is shown in the bottom panel of **Figure 9**.

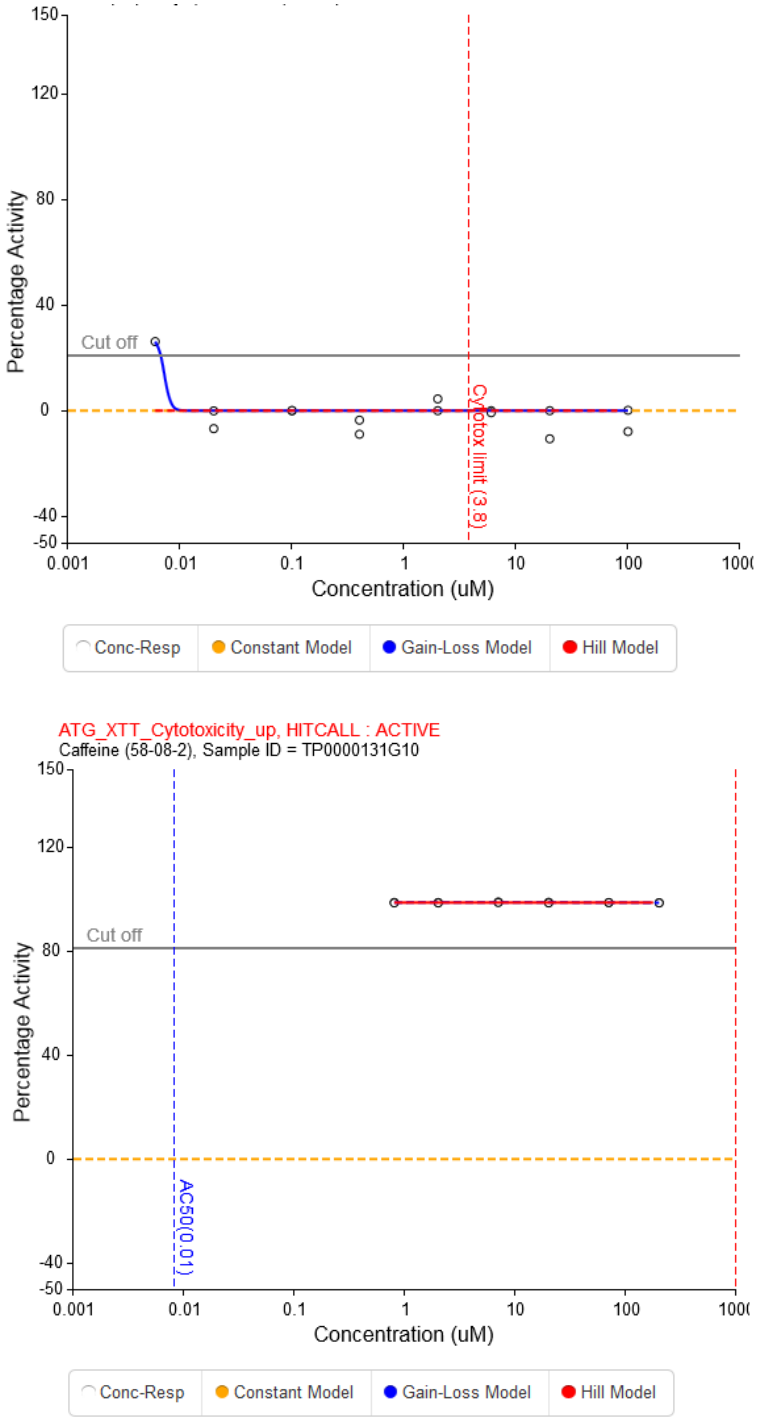

Figure 9. Examples of poorly determined AC50 values. Top – the calculated AC50 is 0.000365 µM and the lowest concentration tested is 0.006 µM. Bottom – the calculated AC50 is 0.00826µM and the lowest concentration tested is 0.8 µM.

Comparison to reference chemicals

Comparison to reference chemicals is a common way to assess the validity of *in vitro* assays. Reference chemicals are identified for many ToxCast assays (in the Dashboard **Assay Summary** tab and the *Assay_Summary_151020.csv* file), but the high number of unique biological targets in ToxCast means that not every assay has a defined reference. Users may choose to identify reference chemicals relevant to their intended application of the data. Both the potency (AC50) and efficacy (top or max response) of the response of a chemical of interest can be compared to a reference chemical.

For example, the Ceetox assays for a decrease in estrone levels (CEETOX_H295R_ESTRONE_dn) use prochloraz as a control chemical. The curve for triadimefon looks similar to that for Prochloraz (**Figure 10**). The efficiency is also similar, both reaching approximately 60-fold induction (notice the log2 scale of the y-axis). However, comparing the AC50s, prochloraz is nearly 100 fold more potent than triadimefon. This does not necessarily mean that the data for triadimefon is not relevant, a range of potencies for different chemicals is expected, but this is one factor that should be considered, and final interpretation depends on the intended use of the data.

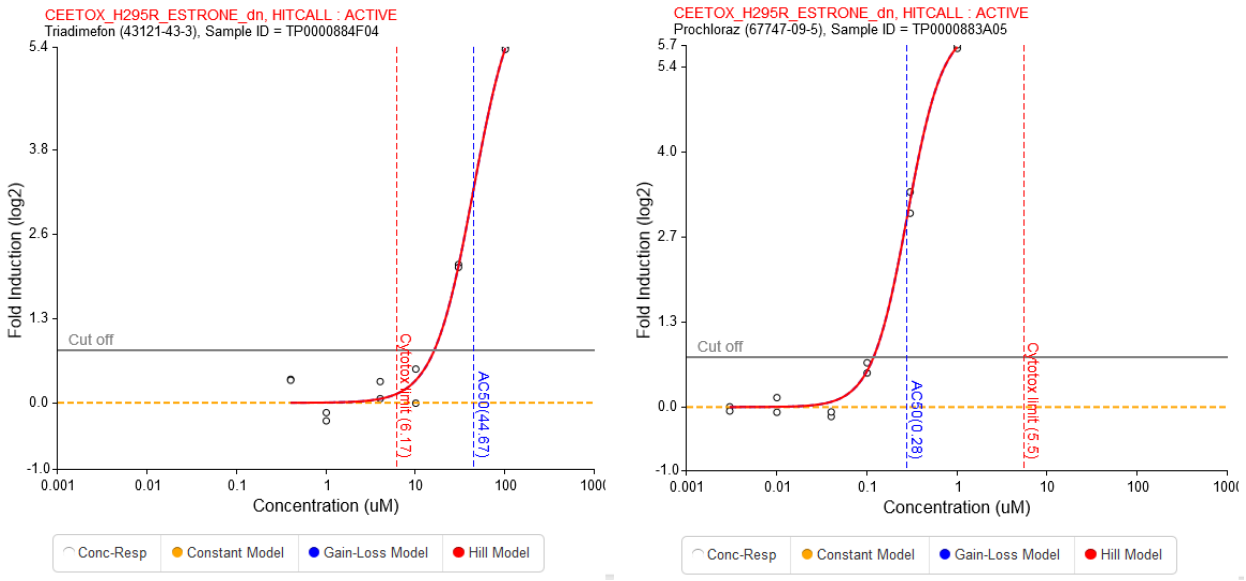

Figure 10. Comparison of an example chemical (triadimefon) to a reference chemical (prochloraz) in the Ceetox assay for decreased estrone levels (CEETOX_H295R_ESTRONE_dn).

Consistency among orthogonal assays and/or related targets

Several biological endpoints are covered by multiple ToxCast assays. Perfect consistency cannot be expected due to the high-throughput nature of the assays, but comparing multiple assays can improve the interpretation and confidence in the data. In addition to reviewing the list of assays and manually identifying assays with similar targets, columns containing information on the assay targets (e.g. *intended_target_gene_name*) in the Assay summary file (*Assay_Summary_151020.csv*) can be sorted or filtered.

To show a specific example for triadimefon, there are seven assays designed to measure mitochondrial membrane potential (MMP) (**Table 3**). In the Apredica (APR) technology, two cell types are used (HepG2 and primary rat hepatocytes ["Hepat"]), and three time points are measured in each. The Tox21 assay measures the MMP in HepG2 cells at 24 hours. Triadimefon is only positive for one of these seven assays, APR_HepG2_MitoMembPot_24h_dn. Even without an analysis of the dose-response curve for this assay, the lack of response in the other assays suggests that this may be a non-specific response. Overall, there is weak support for a specific disruption of mitochondrial membrane potential by triadimefon.

Assay Endpoint Name	AC50
APR_Hepat_MitoFxnI_1hr_dn	NA
APR_Hepat_MitoFxnI_24hr_dn	NA
APR_Hepat_MitoFxnI_48hr_dn	NA
APR_HepG2_MitoMembPot_1h_dn	NA
APR_HepG2_MitoMembPot_24h_dn	33.9
APR_HepG2_MitoMembPot_72h_dn	NA
TOX21_MMP_ratio_down	NA

Table 3. Seven ToxCast assays that measure mitochondrial membrane potential and the results for an example chemical (triadimefon).

Specificity

Specificity may be considered in the context of the promiscuity of an individual assay, or in the context of how many or which assays a single chemical hits. Information regarding the number of chemicals active for a given assay in relation to the total number of chemicals tested can be found in the *Assay_Quality_Summary_Stats_151020.csv* file accessed from the *Download Assay Information* link on the data download page. The column *test* provides the number of samples tested, *acnt* provides the number of active samples and *apct* gives the percent active samples.

If one were to simply sort the assays based on the *apct*, the Novascreen (NVS) assays would clearly have the highest percent actives, up to 95%. However this analysis is quite misleading as the NVS assays were first tested at a single concentration, and only actives from the initial screen were tested in multiple concentrations. The *apct* value is calculated on those chemicals selected for multiple concentration screening. Therefore, the high percent actives reflects the ability of the single concentration screen to identify positive chemicals rather than the true ratio of active chemicals out of all chemicals tested.

Another group of assays with a high number of actives is those related to PXR (ATG_PXRE_CIS_up and CLD gene expression assays for PXR target genes). Many of these assays are active for > 40% of chemicals tested. As PXR is a xenobiotic-sensing nuclear receptor with a wide variety of targets, the high percent actives may truly reflect the promiscuity of the PXR receptor rather than the ToxCast assays designed to measure its activity. Overall, there are no recommended guidelines for what percent active samples represent a "promiscuous" assay, and several factors must be considered, but this information may be helpful on an individual basis.

The other context for specificity is illustrated well by the Attagene (ATG) assays activated by triadimefon. Triadimefon activates 21 different ATG assays, which measure transcription factor activation (**Figure 11 A**). A closer look at the target of each assay shows that the ATG_CMV_CIS_up assay is a background control reporter used to indicate non-specific interference (**Figure 11 B**). This indicates that it may not be possible to distinguish specific activity from this non-specific background activity for curves that resemble the activity in the CMV assay, such as that of the metal response element (MRE) (**Figure 11 C**). The other positive ATG assays for triadimefon should be analyzed in a similar manner. Activation of the CMV background reporter assay does not necessarily mean that all results from ATG are unreliable, however the specificity of each response should be considered with greater scrutiny.

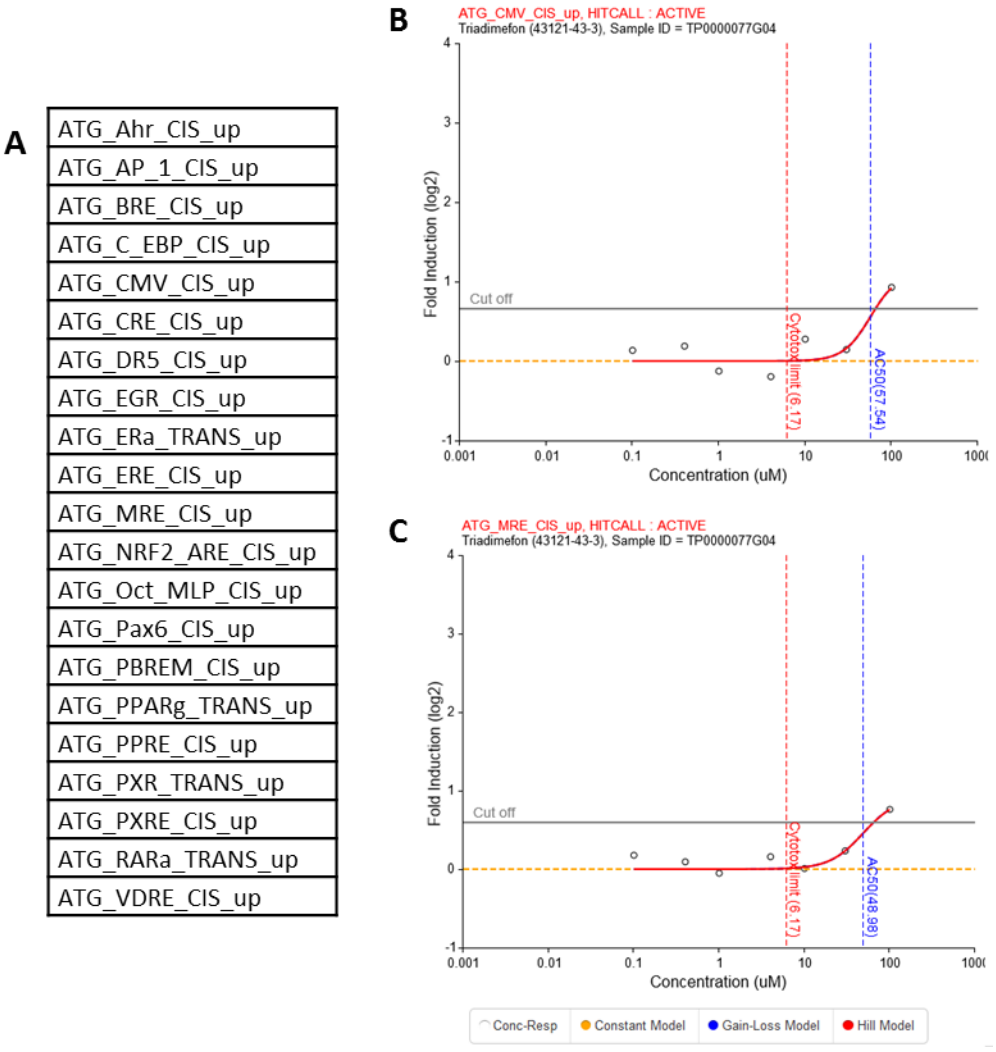

Figure 11. Analysis of the positive results for an example chemical (triadimefon) in the Attagene assays. A – 21 active ATG assays for triadimefon. B – Dose-response curve for CMV, a background control reporter. C – Dose-response curve for the metal response element (MRE) that resembles the activity of CMV.

Interference of cytotoxicity or non-specific cell stress

The ToxCast program recognizes that there is a need to distinguish between toxicity due to the disruption of a specific biomolecular function and toxicity due to general cell stress. This concept has been well described in a recent paper by Judson *et al*.[12], and his analysis of triadimefon is shown in **Figure 12**. ToxCast has built in 35 assays to measure cytotoxicity in various cell types. The full list of these "burst" assays can be obtained by filtering the *Assay_Summary_151020.csv* file for 1's in the *burst_assay* column. In the graphics from Judson *et al*., the median AC50 of the active burst assays (*cytotox median*) is shown in red, and confidence intervals around the median are calculated to give the lower limit (*cytotox min*), shown in the gray box. Any assays with positive results in the cytotoxicity region (gray box) should be carefully analyzed to determine if the activity may be confounded by non-specific cytotoxicity.

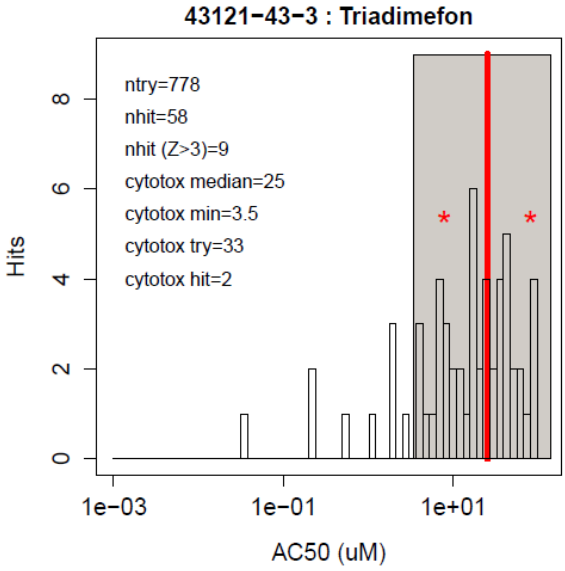

Figure 12. Graph for triadimefon extracted from Judson *et al*. 2016, Supplemental File 4.[12]

For many reasons, it may not be the best practice to use the cytotox limit as a hard filter. The median, and therefore lower limit, are calculated based on active burst assays. As discussed in an earlier section, hit calls may not always be accurate, and the results for burst assays are determined in the same way as all other assays. Therefore, the median could be a misrepresentation of the true chemical response if one or more of the burst assays are potentially mislabeled as active (false positive). The *cytotox min* is based on a statistical calculation with uncertainties. The 35 burst assays do not represent all cell types used in the ToxCast assays, so it cannot be automatically assumed that all cell types would show cytotoxicity in the same concentration range. Although the method for identifying potential interference from cytotoxicity is not perfect, it can be quite effective when considered as a flag for greater scrutiny rather than a filter for relevant assay responses.

[12] Judson, R *et al*. (2016) Analysis of the effects of cell stress and cytotoxicity on in vitro assay activity across a diverse chemical and assay space. *Toxicol Sci*. 152(2):323-39.

It was mentioned above that versioning and release dates are very important for ToxCast, and the cytotoxicity values are a good example of this. The recent Judson *et al*. paper provides an explanation of deriving all the cytotoxicity values, but it is based on the ToxCast data release from 2014. The ToxCast Dashboard provides a "Cytotoxicity Limit (µM)" in the **Chemical Summary** tab which is the cytotox lower bound (lower_bnd_um) from the 2015 data release (most recent). Additional parameters such as the number of active burst assays (nhit) and the cytotoxicity median (cyto_pt_um) are included in a summary file (*cyto_dist_Matrix_151020.csv*). Without regard for the different versions of data, different numerical values for the same chemical in publications versus the Dashboard could be a source of confusion.

Conclusion

After considering the above points, the level of plausibility for each hit call can be determined; i.e. how likely is it that this hit call defined by ToxCast represents true biological activity? The list of active assays can then be refined to plausible active assays. A careful analysis considering at minimum, but not limited to, the points above should improve the confidence in any predictions or conclusions drawn for a chemical from the ToxCast data (e.g. biological targets, molecular initiating events, etc.).