

# Analysis of Quantitative High Throughput Screening Data for Applications in Toxicology

K. R. Shockley<sup>1</sup>, G. E. Kissling<sup>1</sup>, M. Xia<sup>2</sup>, R. Huang<sup>2</sup>, C.P. Austin<sup>2</sup>, and R.R. Tice<sup>1</sup>.

<sup>1</sup>The National Institute of Environmental Health Sciences/National Toxicology Program, NIH, DHHS, Research Triangle Park, NC 27709

<sup>2</sup>NIH Chemical Genomics Center, NIH, DHHS, Bethesda, MD 20892

## Abstract

Quantitative high throughput screening (qHTS) assays play an important role in the Tox21 community efforts to advance toxicology from a predominantly observational science at the level of disease-specific models to a predominantly predictive science based on broad inclusion of target-specific, mechanism-based, biological observations. However, the analysis of qHTS data has largely been motivated by the conservative focus of pharmaceutical applications (i.e., minimizing the risk of Type 1 error) and generally has relied on heuristics rather than statistical tests to make activity calls. To integrate statistical significance with toxicological relevance within qHTS studies, we fit normalized concentration-response data from twelve different agonist nuclear receptor assays (AR, ER, FXR, GR, LXR, PPAR $\alpha$ , PPAR $\gamma$ , RXR, TR $\beta$ , VDR, PXR-human, PXR-rat) for the ToxCast™ 320 compounds to four-parameter Hill equations. An overall F-test comparing the best fit to the Hill equation and a horizontal line (no response) for each substance, using different significance thresholds, was used to identify active compounds within the tested concentration range. Compounds active below the minimum tested concentration were found by comparing the distribution of measured responses to a control value. We compare this statistical method to a previously utilized heuristic approach.

## Introduction

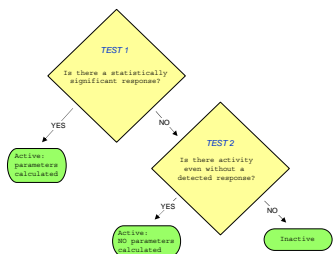
Our goal is to develop a general statistical framework to evaluate qHTS data for toxicological testing. Data was generated using  $\beta$ -lactam coupled coumarin-fluorescein substrate reporter assays (all assays except PXR) or luciferase assays (PXR assays) in order to screen for nuclear receptor agonist activity. Raw plate reads for each titration point were first normalized relative to the positive control response (i.e., 100% response) and the basal response in the DMSO only wells (i.e., 0%), and then corrected by applying a pattern correction algorithm using the compound-free control plates (DMSO plates). Outliers are identified and removed based on the fit to the Hill equation. The curve class identification procedure was modified from a previous approach (Inglese et al., 2006):

| Curve Class | Curve Description | Efficacy | r <sup>2</sup> | Asymptotes | Infection | Activity Designation |
|-------------|-------------------|----------|----------------|------------|-----------|----------------------|
| 1.1         | Complete          | >80%     | ≥0.9           | 2          | Yes       | ACTIVE               |
| 1.2         | Partial           | ≤80%     | ≥0.9           | 2          | Yes       | ACTIVE               |
| 1.3         | Complete          | >80%     | <0.9           | 2          | Yes       | ACTIVE               |
| 1.4         | Partial           | ≤80%     | <0.9           | 2          | Yes       | INCONCL              |
| 2.1         | Incomplete Curves | >80%     | >0.9           | 1          | Yes       | ACTIVE               |
| 2.2         |                   | ≤80%     | >0.9           | 1          | Yes       | INCONCL              |
| 2.3         |                   | >80%     | <0.9           | 1          | Yes       | INCONCL              |
| 2.4         |                   | <80%     | <0.9           | 1          | Yes       | INCONCL              |
| 3           | Single Point      | >min     | NA             | 1          | No        | INCONCL              |
| 4           | Inactive          | NA       | NA             | 0          | No        | INCONCL              |

modified from Inglese et al., 2006

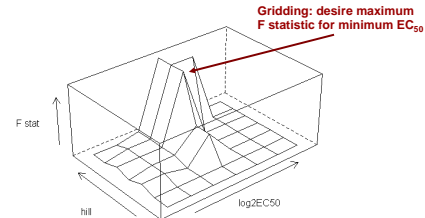
## Testing Approach

The normalized and filtered data is then evaluated for bioactive compound activity using the two-tiered strategy below:



## Figure 2. Test 2

$$t = \frac{(\bar{x} - 0)}{\frac{s}{\sqrt{n}}} \rightarrow \text{p-value} \xrightarrow{\text{multiple test correction}} \text{corrected p-value}$$



## Figure 1. Test 1

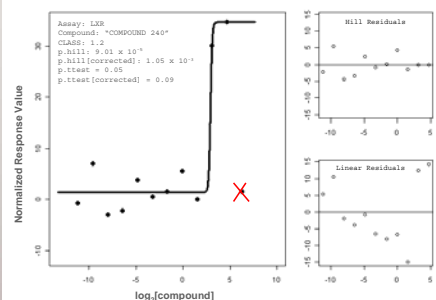
Model 1: Response = Concentration

$$\text{Model 2: Response} = E_{\text{min}} + \frac{(E_{\text{max}} - E_{\text{min}})}{1 + \frac{2 \log_2 EC_{50}}{\text{Concentration}}}$$

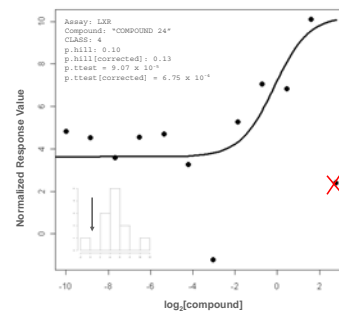
$$F = \frac{(RSS_1 - RSS_2)}{RSS_2} \rightarrow \text{p-value} \xrightarrow{\text{multiple test correction}} \text{corrected p-value}$$

## Results

### Figure 3. Test 1 "Active"



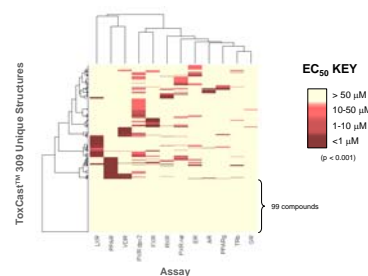
### Figure 4. Test 2 "Active"



## Table 1.

|               | HEURISTICS TEST | TEST 1   |          |           | TEST 2   |          |           | OVERLAP WITH HEURISTICS |          |           |
|---------------|-----------------|----------|----------|-----------|----------|----------|-----------|-------------------------|----------|-----------|
|               |                 | p < 0.05 | p < 0.01 | p < 0.001 | p < 0.05 | p < 0.01 | p < 0.001 | p < 0.05                | p < 0.01 | p < 0.001 |
| AR            | 0               | 57       | 3        | 1         | 43       | 26       | 9         | 0                       | 0        | 0         |
| ER            | 13              | 146      | 95       | 53        | 1        | 0        | 0         | 13                      | 12       | 12        |
| FXR           | 2               | 85       | 49       | 19        | 22       | 29       | 16        | 2                       | 2        | 2         |
| GR            | 1               | 53       | 12       | 5         | 8        | 1        | 0         | 0                       | 0        | 0         |
| LXR           | 15              | 117      | 66       | 14        | 60       | 51       | 32        | 13                      | 11       | 8         |
| PPAR          | 0               | 42       | 26       | 3         | 16       | 25       | 39        | 0                       | 0        | 0         |
| PPAR $\alpha$ | 4               | 44       | 27       | 13        | 23       | 11       | 2         | 4                       | 3        | 2         |
| RXR           | 4               | 91       | 63       | 31        | 12       | 18       | 8         | 4                       | 4        | 4         |
| TR $\beta$    | 3               | 66       | 26       | 9         | 17       | 11       | 2         | 2                       | 2        | 2         |
| VDR           | 2               | 33       | 17       | 10        | 30       | 27       | 26        | 2                       | 2        | 2         |
| PXR-dpx2      | 62              | 198      | 171      | 139       | 0        | 0        | 0         | 48                      | 48       | 46        |
| PXR-rat       | 26              | 125      | 94       | 70        | 15       | 9        | 1         | 15                      | 15       | 15        |
| TOTAL         | 103             | 298      | 278      | 209       | 249      | 193      | 113       | 243                     | 178      | 76        |

### Figure 5. Potency of Actives



## Discussion

Finding candidates for the large numbers of compounds that need to be tested is part of the long-range vision for toxicity testing (Bucher et al., 2008). Here, we examined the responses of the 309 unique structures from the ToxCast™ 320 compounds on nuclear receptors involved in reproduction and development (AR, ER), bile acids and xenobiotic metabolism (FXR, PXR, VDR), and lipid metabolism and energy homeostasis (GR, LXR, PPAR, PPAR $\gamma$ , RXR, TR $\beta$ ). A total of 298 out of the 309 structures showed activity in at least one assay ( $p < 0.05$ ). Agonist activity in these steroid receptors could indicate that a compound can adversely drive the expression of genes, which may lead to endocrine-related diseases such as cancer, cardiovascular disease and inflammation (Wilkinson et al., 2008). Designating actives based on the conservative curve class method (Inglese et al., 2006) found many fewer candidates than using an automated procedure based on a statistical approach. Traditional methods used to assess the significance of non-linear regression analyses rely heavily on human scrutiny of graphical diagnostics (e.g., inspection of residual plots or comparisons of the fit and the raw data), which are not feasible in the qHTS analysis context with thousands of compounds. Furthermore, confounding effects such as autofluorescence and cytotoxicity may complicate the generation of data and interpretation of results. Computational considerations such as widespread gridding of initial value estimates and multiple test corrections also need to be considered. Finally, care should be taken for removing outliers.

## Conclusions and Future Work

The overall F-test can provide a larger number of potentially toxic candidates than heuristic approaches.

- Q1: Can the approach be extended to compare data generated in the same assay?
- Q2: What is the best way to compare the potency of compounds?
- Q3: What is the sensitivity and specificity of the curve class detection?
- Q4: How should antagonists be detected?

## References

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