Adaptation of the BG1Luc Estrogen Receptor Transactivation Test Method to qHTS: Comparison of Results from Both Methods

P Ceger[1], J Strickland[1], L Rinckel[1], W Casey[2]

[1]ILS, Inc., RTP, NC, USA; [2]NICEATM/NTP/HHS, RTP, NC, USA

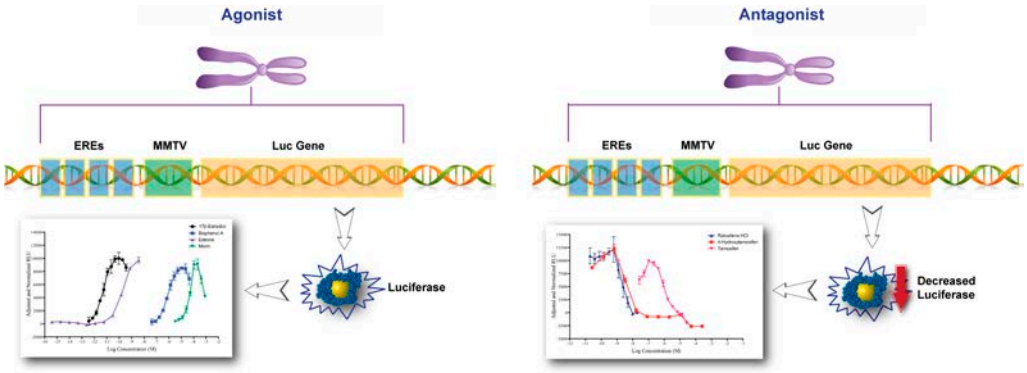
Abstract

In 2011, the National Toxicology Program Interagency Center for the Evaluation of Alternative Toxicological Methods nominated the BG1Luc estrogen receptor (ER) transactivation (TA) test method (BG1Luc ER TA) to Tox21 to be adapted into a quantitative high-throughput screening (qHTS) format. The Tox21 collaboration, an effort by the National Toxicology Program, NIH Chemical Genomics Center, Environmental Protection Agency, and Food and Drug Administration, was formed to advance toxicity testing by shifting from traditional *in vivo* tests to *in vitro* methods. A major goal of Tox21 is to prioritize chemicals for in-depth toxicity testing. One approach for prioritization is to use qHTS cell- and biochemical-based assays to construct concentration–response curves for thousands of chemicals. The Tox21 consortium adapted the BG1Luc ER TA method to a qHTS format. Data were generated for approximately 10,000 chemicals with both the agonist and antagonist versions of the qHTS method. Seventy-six chemicals were tested with both the BG1Luc ER TA manual and qHTS methods. These data were used to evaluate the degree to which classifications of test chemicals with the manual and qHTS methods matched the classifications for performance standards (accuracy) and the degree to which the classifications were identical between the two methods (concordance). Agonist and antagonist methods produced 97% to 100% accuracy and 93% to 96% concordance, respectively, demonstrating that the performance of the qHTS format is comparable to that of the validated BG1Luc ER TA method. (ILS staff supported by NIEHS contract N01-ES 35504.)

Introduction

- The LUMI-CELL® BG1Luc4E2 estrogen receptor (ER) transactivation (TA) test method (BG1Luc ER TA) detects estrogen receptor agonists and antagonists.
- The method uses BG-1Luc 4E2 cells (Rogers and Denison 2000):
  - An immortalized human ovarian adenocarcinoma cell line
  - Stably transfected with an estrogen-responsive luciferase reporter gene
  - Measures TA via ER-mediated pathways (**Figure 1**)

Figure 1 Overview of BG1Luc ER TA Agonist and Antagonist Protocols



Adaptation of the BG1Luc ER TA Manual Method to qHTS

- Adaptation to qHTS was conducted in a phased approach.
- Methods submitted to Tox21 must be adapted to a 1536-well format.
- Guidance criteria for Tox21 assays are listed on the NCATS website: http://www.ncats.nih.gov/research/reengineering/ncgc/assay/criteria/criteria.html.
- Tox21 assays are evaluated using a small library such as the Sigma-Aldrich Library of Pharmacologically Active Compounds, run in triplicate (BSC Review).
- Assay acceptance criteria include a Z factor (Zhang 1999) greater than 0.5, a coefficient of variation less than 10%, and a signal-to-background ratio larger than 3 (NTP 2010).
- Assays that meet these acceptance criteria are used to test the Tox21 10,000-chemical library.
- Having met these performance criteria, the BG1Luc ER TA (BG1) qHTS method was then considered to be adapted for Tox21.

Table 1 Comparison of BG1Luc ER TA Manual and qHTS Methods

BG1 Manual	BG1 qHTS
Hand pipetted	Fully automated
96-well plate	1536-well plate
2 test substances per plate	1408 test substances per plate
11 test substance concentrations per plate	One test substance concentration per plate
Complete concentration-response curve for each substance on a single plate	Complete concentration-response curve generated over 15 test plates
Concentrations determined by range finder, followed by focused testing (~3 log range) up to the limit of solubility or 1 mM	Fixed concentrations typically ranging from 0.5 nM to 92 μM
Each test substance tested in triplicate in each experiment	Each test substance tested once in each experiment
Each experiment performed at least twice (OECD 2012a,b)	Each experiment performed in triplicate
40,000 cells per well	4000 cells per well
200 μL per well	10 μL per well
Wash steps	No wash steps
7 to 14 days	3 days
Viability determined visually	Viability determined by fluorescence

Figure 2 BG1 qHTS Test Plate Layout

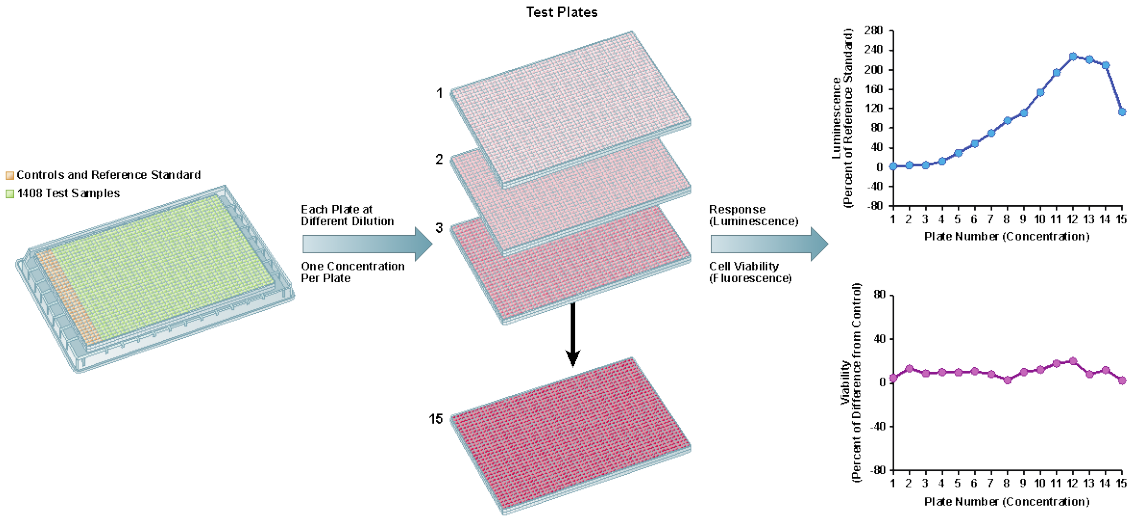


Figure represents one set of 15 plates.

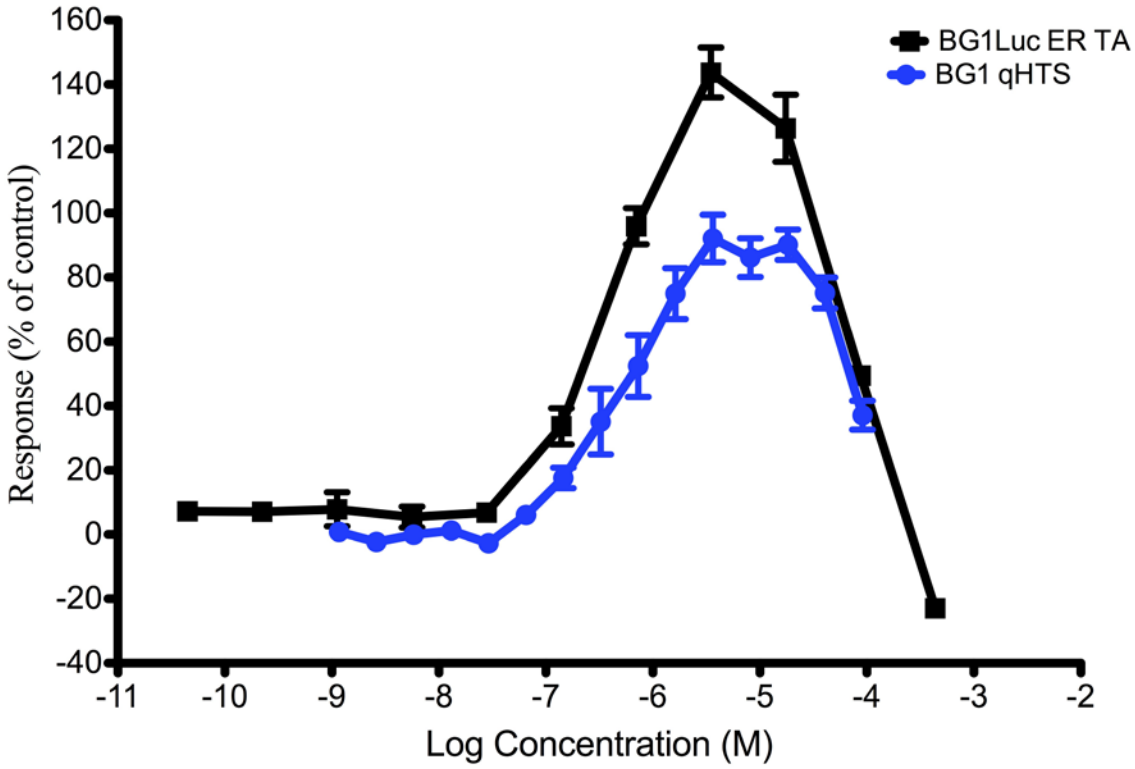
- In the BG1 qHTS method, concentration-response data are generated for all chemicals by testing in fifteen 1536-well test plates (**Figure 2**).
- The 10,000 chemicals in the Tox21 library are tested simultaneously by testing 10 sets of 15 test plates (Inglese 2005) for a total of 150 plates per experiment.
- Each experiment is repeated in three independent runs.
- The complete set of 10,000 Tox21 chemicals has been screened using the BG1 qHTS method.
- Data analyses are ongoing.
- Seventy-six chemicals were tested with both the BG1 manual and qHTS methods, allowing for comparison of data between methods.

Data Analysis Methods

- Concentration-response curves were graphed for each substance. A positive, negative, or inconclusive classification was assigned to each graph.
- The BG1 qHTS data were graphed for comparison to the BG1 manual method (**Figures 3** and **4**).
- Each graph was evaluated and assigned a classification of positive, negative, or inconclusive.

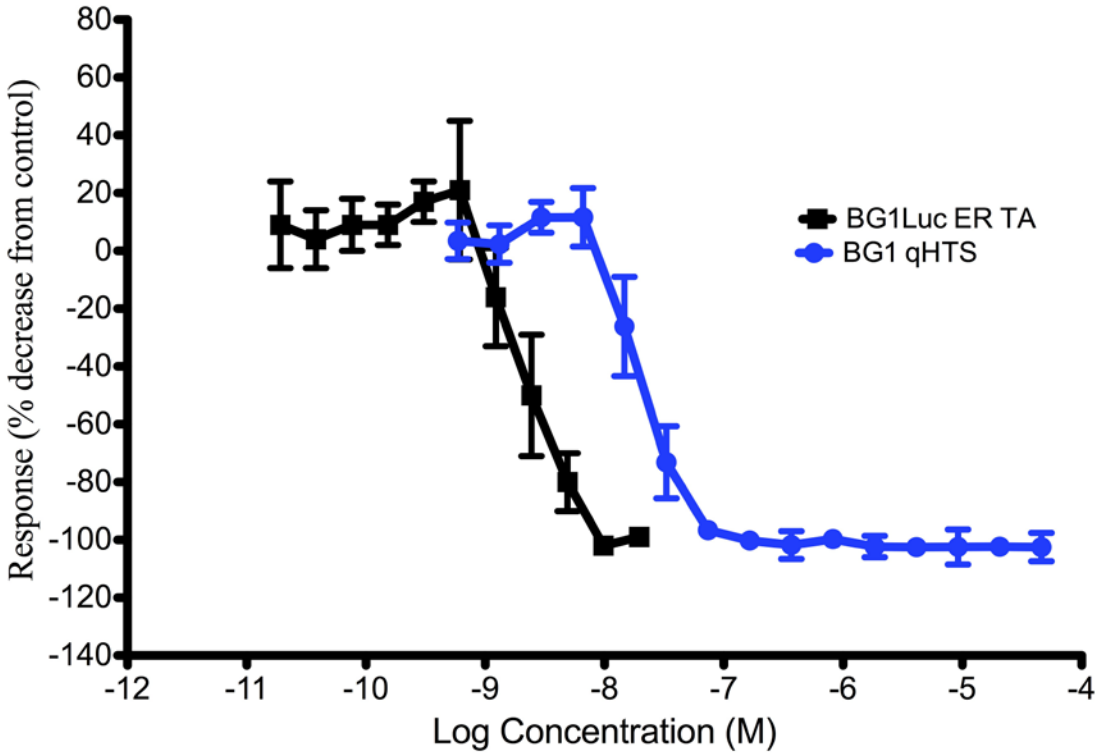
- For positive substances, the half-maximal effective concentration (EC_{50}) or the half-maximal inhibitory concentration (IC_{50}) values were calculated using a 4-parameter Hill function.

Figure 3 Representative Positive Agonist Response with BG1Luc ER TA Manual and BG1 qHTS Methods: Bisphenol A



The graph shows concentration–response curves for bisphenol A tested with the agonist protocols for the BG1Luc ER TA manual and qHTS methods. Each data point represents the mean ± standard deviation from the mean for three experiments. These curves were classified as positive.

Figure 4 **Representative Positive Antagonist Response with BG1Luc ER TA Manual and BG1 qHTS Methods: Raloxifene HCl**



The graph shows concentration–response curves for raloxifene HCl tested with the antagonist protocols for the BG1Luc ER TA manual and qHTS methods. Each data point represents the mean ± standard deviation from the mean for three experiments. These curves were classified as positive.

Accuracy and Concordance

- ICCVAM developed performance standards for the BG1Luc ER TA manual method to evaluate the comparability of proposed test methods that are functionally and mechanistically similar (ICCVAM 2011). The performance standards include a minimum list of reference substances for assessing the accuracy of the proposed test method (42 for agonists; 25 for antagonists).

Accuracy

- Accuracy was determined for both test methods using the reference substances from the ICCVAM performance standards.
    - Substances that were classified as inconclusive with either method were omitted from analysis.

Table 2 Accuracy of the Agonist Protocols for the BG1Luc ER TA Manual and qHTS Methods

	BG1 Manual	BG1 qHTS
Positive	27	26
Negative	7	7
Overall	34/34 (100%)	33/34 (97%)

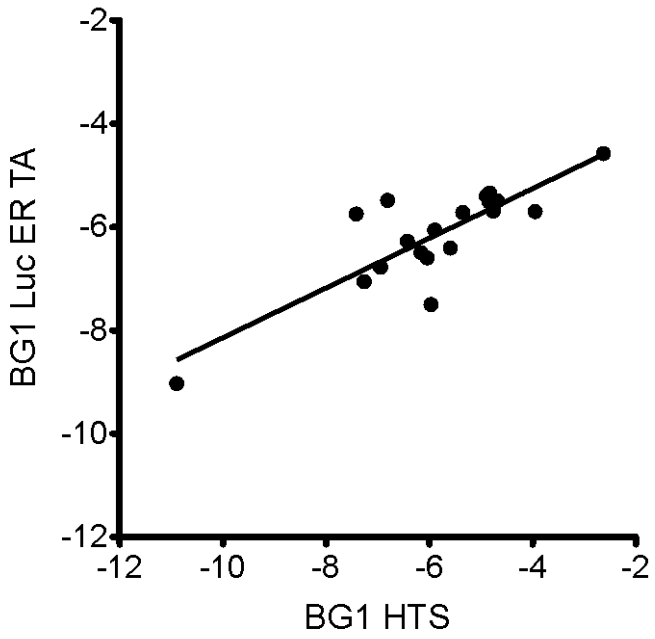
- Agonist accuracy was calculated for 34 substances (27 positive, 7 negative).
- Accuracy for the BG1 manual method was 100% (34/34).
- Accuracy for the BG1 qHTS method was 97% (33/34).

Table 3 Accuracy of the Antagonist Protocols for the BG1Luc ER TA Manual and qHTS Methods

	BG1 Manual	BG1 qHTS
Positive	3	3
Negative	22	22
Overall	25/25 (100%)	25/25 (100%)

- Antagonist accuracy was calculated for 25 substances (3 positive, 22 negative).
- Accuracy for both methods was 100% (25/25).
- An EC_{50} or IC_{50} value was calculated for all positive substances from the concentration-response curves for the BG1 manual and qHTS methods.
- Linear regression analysis was used to compare the two value sets (**Figures 5** and **6**).

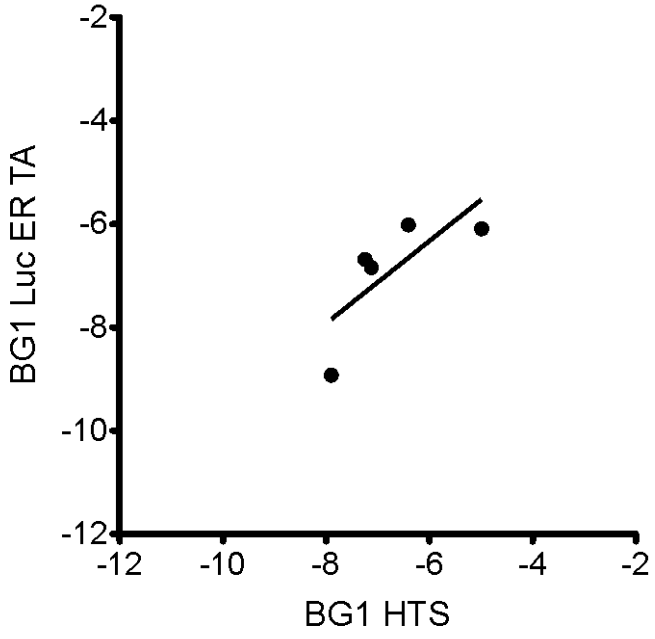
Figure 5 Linear Regression Analysis of BG1Luc ER TA Manual and qHTS EC_{50} Values



The line represents the linear regression through those EC_{50} values.

- The slope of the linear regression line is 0.48, r^2 is 0.69.

Figure 6 Linear Regression Analysis of BG1Luc ER TA Manual and qHTS IC_{50} Values



The line represents the linear regression through those IC_{50} values.

- The slope of the linear regression line is 0.80, r^2 is 0.56.

Concordance

- The results from the 76 chemicals tested with both methods were evaluated for concordance.
  - Substances that were classified as inconclusive with either method were omitted from analysis.

Table 4 Concordance of the Agonist Protocols for the BG1Luc ER TA Manual and qHTS Methods

- Concordance was calculated for 61 substances (35 positive, 26 negative).

		BG1 qHTS Classification		
		Positive	**Negative**	**Total**
BG1 Manual Classification	**Positive**	31	4	35
	Negative	0	26	26
	Total	31	30	61

- Concordance between the BG1 manual and BG1 qHTS methods was 93% (57/61).

Table 5 Concordance of the Antagonist Protocols for the BG1Luc ER TA Manual and qHTS Methods

- Concordance was calculated for 73 substances (9 positive, 64 negative).

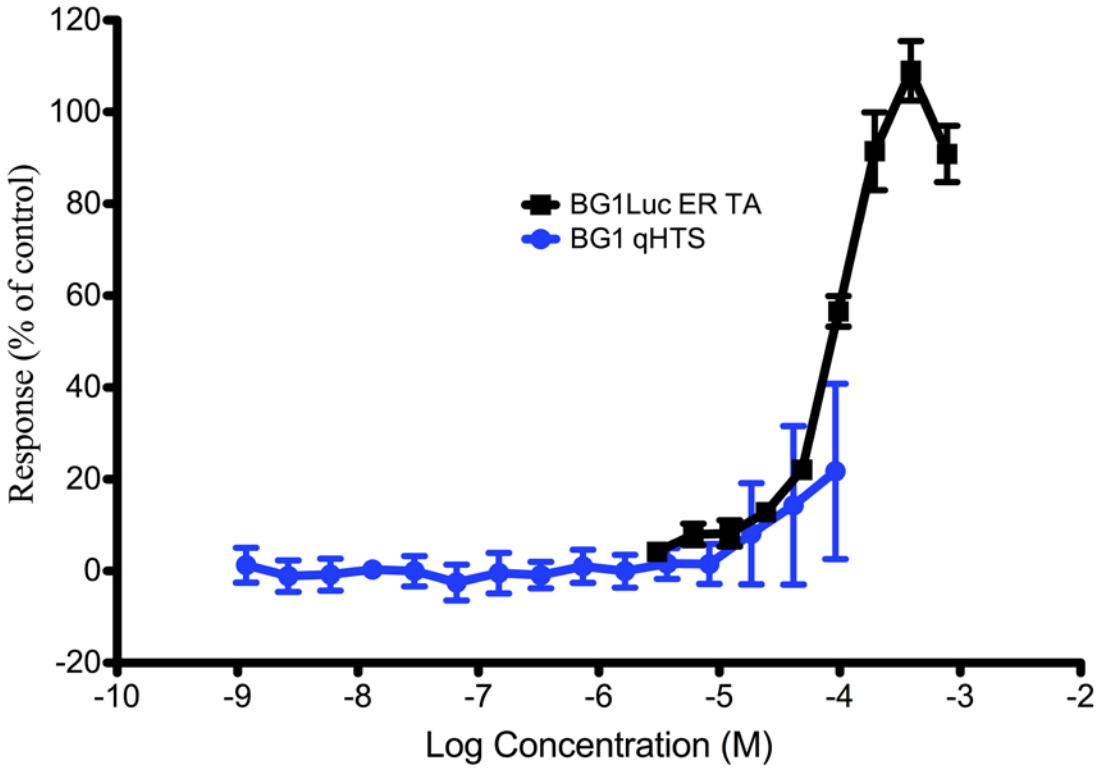
		BG1 qHTS Classification		
		Positive	**Negative**	**Total**
BG1 Manual Classification	**Positive**	6	3	9
	Negative	0	64	64
	Total	6	67	73

- Concordance between the BG1 manual and qHTS methods was 96% (70/73).

Discussion

- Accuracy was determined by comparing the results from each method with ICCVAM reference classifications for the 34 substances used to evaluate agonists and the 25 substances used to evaluate antagonists. Performance standards include reference substances that are recommended for evaluating functionally and mechanistically similar test methods (ICCVAM 2011).
- Using the performance standards, the accuracy of the BG1 qHTS was nearly identical to that of the BG1 manual method and provided comparable EC_{50} and IC_{50} values.
- Concordance between the two methods was high, with the few discrepancies appearing to be related primarily to differences in the upper limit of testing concentrations (see **Figures 7** and **8** for examples).

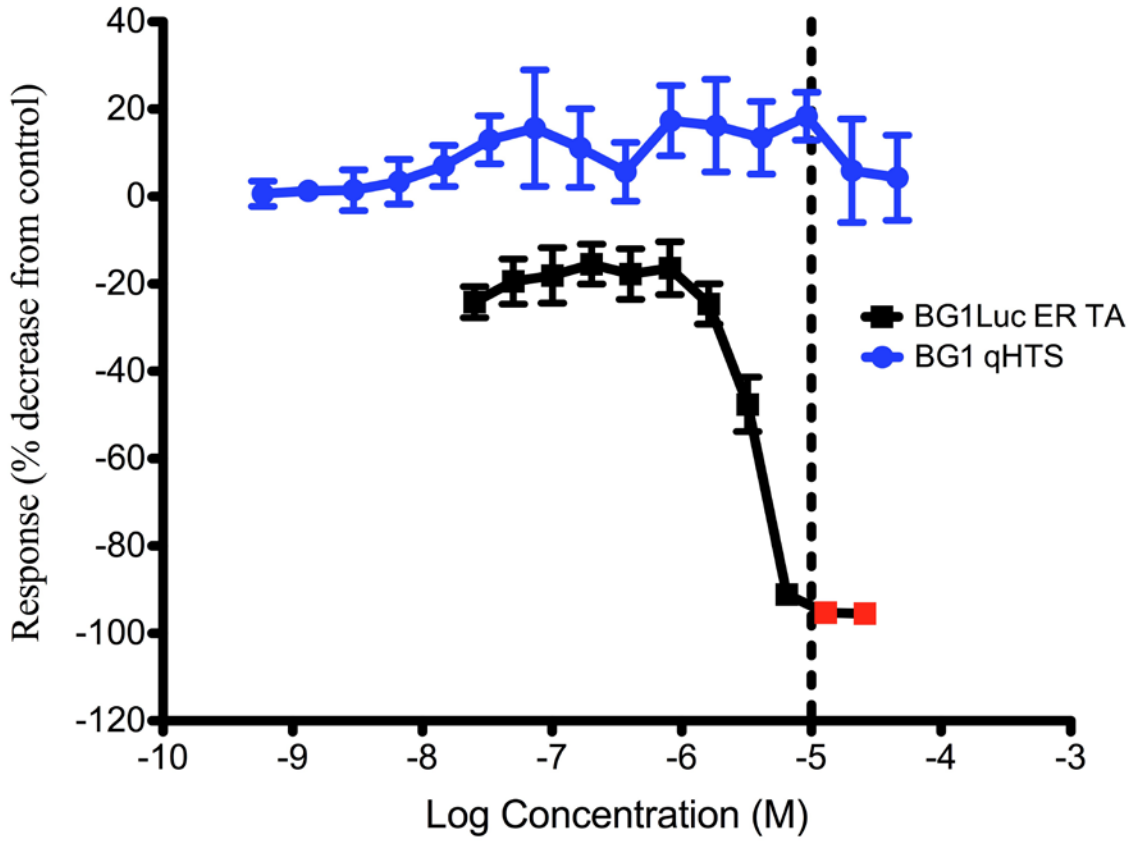
Figure 7 Discordant Agonist Response with BG1Luc ER TA Manual and qHTS Methods: Phenolphthalein



Each data point represents the mean ± standard deviation from the mean for three experiments. The BG1Luc ER TA manual curve was classified as positive; the BG1 qHTS curve was classified as negative.

- The dashed line indicates the highest concentration tested with the BG1 qHTS method.
- Increased variability among the three experimental replicates indicates that the substance tested positive in at least one experiment.
- A general upward trend suggests that this substance would test positive with the BG1 qHTS method if tested at higher concentrations.

Figure 8 **Discordant Antagonist Response with BG1Luc ER TA Manual and qHTS Methods: Medroxyprogesterone Acetate**



Each data point represents the mean ± standard deviation from the mean for three experiments. Points highlighted in red indicate where cytotoxicity likely impacted the response. The BG1Luc ER TA manual curve was classified as positive; the BG1 qHTS curve was classified as negative.

- Antagonist substances that show a decrease in response only at concentrations greater than 10 µM (vertical dashed line) are considered negative (ICCVAM 2011).

Conclusions

- Accuracy for the BG1 qHTS was nearly identical to that of the BG1 manual method (97% [33/34] and 100% [25/25] for the agonist and antagonist protocols, respectively), with comparable EC_{50} and IC_{50} values.
- Concordance between the BG1 manual and qHTS methods was 93% (57/61) and 96% (70/73) for the agonist and antagonist protocols, respectively. The few discordant values with the BG1 qHTS method were due to negative results for substances that tested positive with the BG1 manual method and may be attributed to lower maximum concentrations tested with the qHTS method.

- The BG1 qHTS has performed well for a subset of ICCVAM reference substances.

References

ICCVAM. 2011. ICCVAM Test Method Evaluation Report. The LUMI-CELL® ER (BG1Luc ER TA) Test Method: An In Vitro Assay for Identifying Human Estrogen Receptor Agonist and Antagonist Activity of Chemicals. NIH Publication No. 11-7850. Research Triangle Park, NC:National Institute of Environmental Health Sciences.

Inglese J, Auld DS, Jadhav A, Johnson RL, Simeonov A, Yasgar A, et al. 2006. Quantitative high-throughput screening: a titration-based approach that efficiently identifies biological activities in large chemical libraries. Proc Natl Acad Sci USA 103(31): 11473-11478.

NTP. 2010. Review of the Biomolecular Screening Branch by the NTP Board of Scientific Counselors. Research Triangle Park, NC:National Toxicology Program.

OECD. 2012a. Test No. 455. Performance-Based Test Guideline for Stably Transfected Transactivation In Vitro Assays to Detect Estrogen Receptor Agonists [adopted 2 October 2012]. In: OECD Guidelines for the Testing of Chemicals, Section 4: Health Effects. Paris:OECD Publishing.

OECD. 2012b. Test No. 457. BG1Luc Estrogen Receptor Transactivation Test Method for Identifying Estrogen Receptor Agonists and Antagonists [adopted 2 October 2012]. In: OECD Guidelines for the Testing of Chemicals, Section 4: Health Effects. Paris:OECD Publishing.

Rogers JM, Denison MS. 2000. Recombinant cell bioassays for endocrine disruptors: development of a stably transfected human ovarian cell line for the detection of estrogenic and anti-estrogenic chemicals. In Vitr Mol Toxicol 13(1): 67-82.

Zhang JH, Chung TD, Oldenburg KR. 1999. A simple statistical parameter for use in evaluation and validation of high throughput screening assays. J Biomol Screen 4(2): 67-73.

Acknowledgements

The Intramural Research Program of the National Institute of Environmental Health Sciences (NIEHS) supported this poster. Technical support was provided by ILS, Inc., under NIEHS contract N01-ES 35504.