Performance of the Tox21 BG1Luc and ER β-Lactamase Estrogen Receptor Transactivation Assays

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Abstract

Two estrogen receptor (ER) transactivation assays (BG1Luc and HEK293 ER β-lactamase [ER-Bla]) were adapted for the Tox21 high-throughput screening program. Both assays detect substances with ER agonist (Ag) or antagonist (Ant) activity. BG1Luc endogenously expresses full-length ER and is stably transfected with a luciferase reporter gene. ER-Bla is stably transfected with the human ER-α ligand-binding domain and a β-lactamase reporter gene. Approximately 10,000 chemicals in the Tox21 compound library were tested three times in each assay in Ag and Ant modes. To differentiate antagonism from cytotoxicity, cell viability was determined. Concentration–response data (N=15) were analyzed to evaluate assay performance. Data quality was high in all assays with acceptable signal to background ratio (2.5 to 8), CV (<10.5%), reproducibility (outcomes across 3 runs ≥87%), and Z′ factor (≥0.4). Results were compared to ICCVAM ER transactivation performance standards. Ag accuracy, sensitivity and specificity were, respectively, 97%, 96%, and 100% for BG1 versus 90%, 87%, and 100% for ER-Bla. Ant accuracy, sensitivity, and specificity were all 100% for both assays. Ag EC_{50} reference standard values for estradiol were 30 pM (BG1) and 275 pM (ER-Bla), while Ant IC_{50} reference values for hydroxytamoxifen were 71 nM (BG1) and 6 nM (ER-Bla). Understanding the factors contributing to differences in performance of these assays is critical to their regulatory acceptance and utilization.

Introduction

- In 2007, the National Research Council released the report *Toxicity Testing in the 21st Century: A Vision and a Strategy* (National Research Council 2007). In that report, the authors envisioned toxicity testing as a holistic process that considers risk context, population and exposure data, chemical characterization, and targeted toxicity testing based on knowledge of toxicity pathways, dose–response, and extrapolation modeling.
- In response to this report, the U.S. Tox21 consortium was formed. Tox21 integrates Federal resources and expertise from the following offices:
  - Environmental Protection Agency
  - National Institutes of Environmental Health Sciences/National Toxicology Program
  - National Institutes of Health (NIH)/NIH Center for Advancing Translational Sciences
  - Food and Drug Administration
- The mission of Tox21 is to research, develop, validate, and translate innovative chemical testing methods for the characterization of toxicity pathways. The use of robotics platforms to screen thousands of chemicals provides a cost-effective approach to prioritize further testing of potentially toxic chemicals.
- One area of particular interest for Tox21 is toxicity associated with the endocrine system, since exposure to "endocrine active chemicals" (EACs) may result in developmental or reproductive problems.
- EACs may affect growth and development through a variety of mechanisms. One such mechanism is estrogenic signaling. Estrogenic signaling pathways are well-characterized and a number of test methods that target them have been developed.
- Two estrogen receptor (ER) transactivation assays, the BG1Luc4E2 (BG1Luc) and the HEK293 ER β-lactamase (ER-Bla), have been adapted to a high throughput screening (HTS) platform and incorporated into the Tox21 program.

Table 1. Overview of Differences Between the Methods

	BG1Luc HTS	ER-Bla HTS
Cell Line	BG-1Luc4E2	HEK293
Tissue of Origin	Ovary	Kidney
Receptor Expression	Native	Stably transfected
Receptors	ER-α and ER-β	ER-α ligand binding domain
Reporter	Luciferase	β-Lactamase
Viability Detection	Fluorescent	Luminescent

Use of the Assays to Screen the Tox21 Chemicals

- Both assays have agonist and antagonist modes.
- The complete set of 10,000 Tox21 chemicals was screened using both assays in agonist and antagonist modes.
  - The 10,000 chemicals were divided among nine master plates, with one test chemical per well. Eighty-eight chemicals were duplicated on all nine master plates.
  - Each master plate was serially diluted 15 times so that each chemical was tested in 15-point serial dilution (5pM–92nM), one plate per dilution.
  - Each experiment consisted of testing 153 plates (9 sets of 15 test plates plus DMSO blanks).
  - Each experiment was repeated three times.
- Cell viability was simultaneously evaluated in each assay to distinguish antagonism from cytotoxicity.

Data Quality

- Data quality was evaluated in several ways:
  - Computation of metrics including signal-to-background detection ratio, coefficient of variation, and Z′ factor (Zhang 1999)
  - Comparison to reference standard values
  - Comparison of 88 chemicals duplicated on every test plate (intra-assay)
  - Comparison of outcome matches across three runs (inter-assay)

Table 2. Agonist Data Quality

		BG1Luc HTS[a]	ER-Bla HTS[a]
Signal-to-background and Z′ factor	Signal-to-background ratio	2.5 ± 0.3	4.6 ± 0.6
	Coefficient of variation (%)	10.3 ± 5.9	4.7 ± 3.7
	Z′ factor	0.5 ± 0.25	0.53 ± 0.09
Reference Standard Values	Estradiol EC_{50} (pM)[b]	30 ± 70	275 ± 80
Intra-assay	EC_{50} correlations (R^2)[c]	0.80	0.83
Inter-assay	Active match (%)	16	7
	Inactive match (%)	87	71
	Fold difference in AC_{50} among three experiments[d]	1.5	1.4

[a] All values are reported as mean values. Standard deviation is reported where applicable.
[b] EC_{50} is the half-maximal effective concentration.
[c] Intra-assay R^2 values were calculated for all positive test substances.
[d] AC_{50} is the half-maximal activity concentration (Inglese 2006).

Table 3. Antagonist Data Quality

		BG1Luc HTS[a]	ER-Bla HTS[a]
Signal-to-background and Z′ factor	Signal-to-background ratio	8.0 ± 0.9	3.3 ± 0.8
	Coefficient of variation (%)	6.5 ± 2.8	5.1 ± 2.8
	Z′ factor	0.8 ± 0.07	0.4 ± 0.1
Reference Standard Values	4-Hydroxytamoxifen IC_{50} (nM)[b]	70.8 ± 12.4	5.8 ± 3.8
Intra-assay	IC_{50} correlations (R^2)[c]	0.76	0.47
Inter-assay	Active match (%)	12	10
	Inactive match (%)	80	78
	Fold difference in AC_{50} among three experiments[d]	1.5	1.5

[a] All values are reported as mean values. Standard deviation is reported where applicable.
[b] IC_{50} is the half-maximal inhibitory concentration.
[c] Intra-assay R^2 values were calculated for all positive test substances.
[d] AC_{50} is the half-maximal activity concentration (Inglese 2006).

Comparison to ICCVAM Performance Standards

- The U.S. National Toxicology Program Interagency Center for the Evaluation of Alternative Toxicological Methods coordinated an international validation study of the BG1Luc assay for the Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM). The validation was completed in 2010.
- A test method evaluation report (ICCVAM 2011) summarizing the study contained performance standards for developing functionally and mechanistically similar test methods as well as for demonstrating proficiency in the BG1Luc assay.
- The ICCVAM performance standards describe:
  - The principles and expected performance of the BG1Luc manual assay
  - Criteria for data interpretation
  - Reference substances for both agonist and antagonist mode, with expected positive and negative outcomes for each substance
- HTS data for test chemicals were reviewed and classified as positive, negative, or inconclusive. For a test substance to be classified as positive, it needed to have a response greater than or equal to 20% that of the positive control and have a response curve that is semi-sigmoidal in shape.
- Results obtained in the BG1Luc HTS and ER-Bla HTS assays were compared to outcomes specified in the performance standards (**Tables 4–7**).

Agonist Sensitivity and Specificity

Table 4. BG1Luc HTS and ER-Bla HTS Agonist Results Compared to BG1 Manual Performance Standards

Performance Standards Substances.	CAS RN	Performance Standards Classification	BG1 HTS Classification	ER-Bla HTS Classification
17-α Estradiol	57-91-0	POS	POS	POS
17-α Ethinyl estradiol	57-63-6	POS	POS	POS
17-β Estradiol	50-28-2	POS	POS	POS
19-Nortestosterone	434-22-0	POS	POS	POS
4-Cumylphenol	599-64-4	POS	POS	POS
4-*tert*-Octylphenol	140-66-9	POS	POS	POS
Apigenin	520-36-5	POS	POS	POS
Bisphenol A	80-05-7	POS	POS	POS
Bisphenol B	77-40-7	POS	POS	POS
Butylbenzyl phthalate	85-68-7	POS	POS	IC
Chrysin	480-40-0	POS	POS	POS
Coumestrol	479-13-0	POS	POS	IC
Daidzein	486-66-8	POS	POS	POS
Dicofol	115-32-2	POS	NEG	IC
Diethylstilbestrol	56-53-1	POS	POS	POS
Estrone	53-16-7	POS	POS	POS
Ethyl paraben	120-47-8	POS	IC	NEG
Fenarimol	60168-88-9	POS	IC	IC
Genistein	446-72-0	POS	POS	POS
Kaempferol	520-18-3	POS	POS	POS
Kepone	143-50-0	POS	POS	POS
***meso*-Hexestrol**	84-16-2	POS	POS	POS
Methyl testosterone	58-18-4	POS	POS	POS
Norethynodrel	68-23-5	POS	POS	POS
***o,p′*-DDT**	789-02-6	POS	POS	POS
***p-n*-Nonylphenol**	104-40-5	POS	POS	NEG
***p,p′*-Methoxychlor**	72-43-5	POS	POS	NEG
Atrazine	1912-24-9	NEG	NEG	NEG
Bicalutamide	90357-06-5	NEG	NEG	NEG
Corticosterone	50-22-6	NEG	NEG	NEG
Hydroxyflutamide	52806-53-8	NEG	NEG	NEG
Linuron	330-55-2	NEG	NEG	NEG
Phenobarbital	50-06-6	NEG	NEG	NEG

Abbreviations: CAS RN = Chemical Abstracts Service Registry Number®; IC = inconclusive; NEG = negative; POS = positive.

Table 5. Agonist Sensitivity and Specificity for the BG1Luc and ER-Bla Assays

	BG1Luc HTS	ER-Bla HTS
Sensitivity	96% (24/25)	87% (20/23)
Specificity	100% (7/7)	100% (7/7)
Accuracy	97% (31/32)[a]	90% (27/30)[a]

[a] Of the 34 agonist substances in the performance standards, two were omitted in BG1Luc HTS and four were omitted in ER-Bla HTS because they were inconclusive.

Data for Substances With Discordant Results in Agonist Assays

Figure 1. Methoxychlor

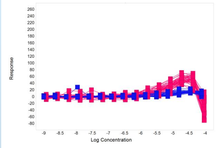


Figure 2.* p-n-*Nonylphenol

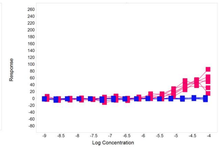


Figure 3. Butylbenzyl Phthalate

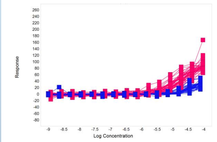


Figure 4. Coumestrol

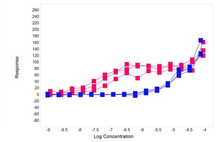
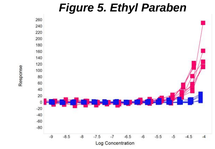


Figure 5. Ethyl Paraben



Response is percent of estradiol control activity.
Red = BG1Luc HTS data. Each line represents a single replicate concentration–response curve.
Blue = ER-Bla HTS data. Each line represents a single replicate concentration–response curve.

- Methoxychlor and *p-n*-nonylphenol were positive in the performance standards and weakly positive in BG1Luc HTS, but negative in ER-Bla HTS (**Figures 1** and **2**).
- Butylbenzyl phthalate and coumestrol were positive in performance standards and in BG1Luc HTS, but were inconclusive in ER-Bla HTS (**Figures 3** and **4**).
  - Although there was an increase in response for both substances, neither substance produced a sigmoidal concentration–response curve when tested in ER-Bla HTS.
  - BG1Luc HTS is more sensitive than ER-Bla HTS. BG1Luc HTS has a reference standard EC_{50} of 30 pM, compared to 275 pM for the ER-Bla HTS.
- Ethyl paraben was positive in the performance standards, inconclusive in BG1Luc HTS, and negative in ER-Bla HTS (**Figure 5**).
  - Although there was an increase in response for ethyl paraben in the BG1Luc HTS, the concentration-response curve was not sigmoidal in shape.
  - When tested in ER-Bla HTS, ethyl paraben showed a slight increase in response at the highest concentration tested, but the response did not reach a minimum threshold of 20%.

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Antagonist Sensitivity and Specificity

- Expected positive and negative outcomes from the ICCVAM performance standards are compared to observed outcomes in the BG1Luc HTS and ER-Bla HTS assays in **Tables 6** and **7**.
- None of the outcomes for either the BG1Luc HTS or the ER-Bla HTS assay was discordant with the performance standards or with the other assay, although four substances yielded inconclusive results with the ER-Bla HTS.

Table 6. BG1Luc HTS and ER-Bla HTS Antagonist Results Compared to BG1 Manual Performance Standards

Performance Standards Substances	CAS RN	Performance Standards Classification	BG1 HTS Classification	ER-Bla HTS Classification
4-Hydroxytamoxifen	68047-06-3	POS	POS	POS
Raloxifene HCl	82640-04-8	POS	POS	POS
Tamoxifen	10540-29-1	POS	POS	POS
17-α Ethinyl estradiol	57-63-6	NEG	NEG	NEG
Apigenin	520-36-5	NEG	NEG	IC
Chrysin	480-40-0	NEG	NEG	NEG
Coumestrol	479-13-0	NEG	NEG	NEG
Genistein	446-72-0	NEG	NEG	IC
Kaempferol	520-18-3	NEG	NEG	IC
Resveratrol	501-36-0	NEG	NEG	IC

Abbreviations: CAS RN = Chemical Abstracts Service Registry Number®; IC = inconclusive; NEG = negative; POS = positive.

Table 7. Antagonist Sensitivity and Specificity for the BG1Luc HTS and ER-Bla HTS Assays

	BG1Luc HTS	ER-Bla HTS
Sensitivity	100% (3/3)	100% (3/3)
Specificity	100% (7/7)	100% (3/3)
Accuracy	100% (10/10)	100% (6/6)[a]

[a] Of the 10 agonist substances in the performance standards, four were omitted in ER-Bla HTS because they were inconclusive.

Conclusions

- BG1Luc HTS and ER-Bla HTS are two ER transactivation assays used in the Tox21 screening program to detect substances that cause ER transactivation.
- While both are ER transactivation assays, they use different cell types, receptors, and reporters.
- Data quality was acceptable in both assays (**Tables 2** and **3**).
- When used to test ICCVAM ER agonist performance standards chemicals, BG1 HTS misidentified only one chemical when a conclusive result was obtained, but two chemicals were inconclusive. All of the ER antagonist performance standards chemicals were correctly identified.
- ER-Bla misidentified three of the ICCVAM ER agonist performance standards chemicals and four more were inconclusive. None of the ER antagonist performance standards chemicals were misidentified when a conclusive result was obtained, but four of them produced inconclusive results.
- These differences may be due to differences in sensitivity in the two assays. Understanding the factors contributing to these differences is critical to their regulatory acceptance and utilization.

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