ICCVAM Recommendations and Limitations of the BG1Luc ER TA Test Method for Identifying Estrogen Receptor Agonists and Antagonists

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Abstract

ICCVAM recently evaluated the BG1Luc estrogen receptor (ER) transactivation (TA) test method. An international interlaboratory validation study was conducted to determine the usefulness and limitations of the BG1Luc ER TA test method as a screening tool to identify substances with *in vitro* ER agonist and antagonist activity. Three laboratories (one each from the United States, Europe, and Japan) tested coded reference chemicals up to three times each. Results were similar across the three participating laboratories. For the agonist protocol, only one of the 35 reference substances that produced a definitive result was discordant (false negative) with existing reference data from other *in vitro* ER TA assays. For the antagonist protocol, all 25 reference substances that produced a definitive result were concordant with existing reference data from other *in vitro* ER TA assays. ICCVAM compared the BG1Luc ER TA test method results with results from the only *in vitro* ER TA test method currently included in national and international regulatory testing guidelines (i.e., U.S. EPA OPPTS 890.1300/OECD Test Guideline 455), resulting in identical accuracy statistics when each method tested the same agonist reference chemicals. ICCVAM concluded that the accuracy of this assay is at least equivalent to that of U.S. EPA OPPTS 890.1300/OECD Test Guideline 455 test method. Thus, the BG1Luc ER TA may be applicable to the U.S. EPA Endocrine Disruptor Screening Program. ICCVAM considered the peer review panel report, public comments, and the comments of the Scientific Advisory Committee on Alternative Toxicological Methods in preparing the ICCVAM final test method recommendations. ICCVAM recommends that the BG1Luc ER TA test method can be used as a screening assay to identify substances with *in vitro* ER agonist and antagonist activity.

Introduction

- The ICCVAM Authorization Act of 2000 (42 U.S.C. 285*l*-3) charged ICCVAM with evaluating the scientific validity of new, revised, and alternative toxicological test methods applicable to the safety testing requirements of U.S. Federal agencies.
- In 2004, Xenobiotic Detection Systems, Inc., nominated the LUMI-CELL® estrogen receptor (ER) transactivation (TA) test method, also known as the BG1Luc ER TA test method, for an interlaboratory validation study. ICCVAM evaluated its status and recommended that the test method be further standardized and validated.
  - The BG1Luc ER TA test method screens substances that may induce (agonism) or inhibit (antagonism) estrogenic activity *in vitro* (**Figure 1**).

  - In the BG1Luc ER TA agonist test method estrogenic substances induce the production of luminescence.
  - In the antagonist test method, anti-estrogenic substances inhibit estrogen-induced luciferase production.

- ICCVAM conducted an international interlaboratory validation study to determine the usefulness and limitations of the BG1Luc ER TA test method as a screening tool to identify substances with *in vitro* ER agonist and antagonist activity.
- This poster summarizes the ICCVAM evaluation and recommendations for the BG1Luc ER TA test method:
  - Usefulness and limitations
  - Test method protocol(s)
  - Future studies
  - Performance standards

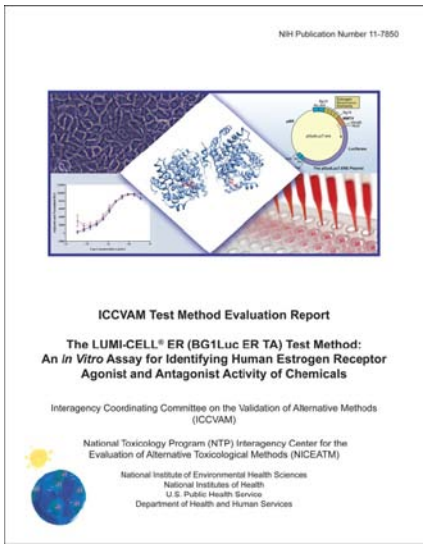
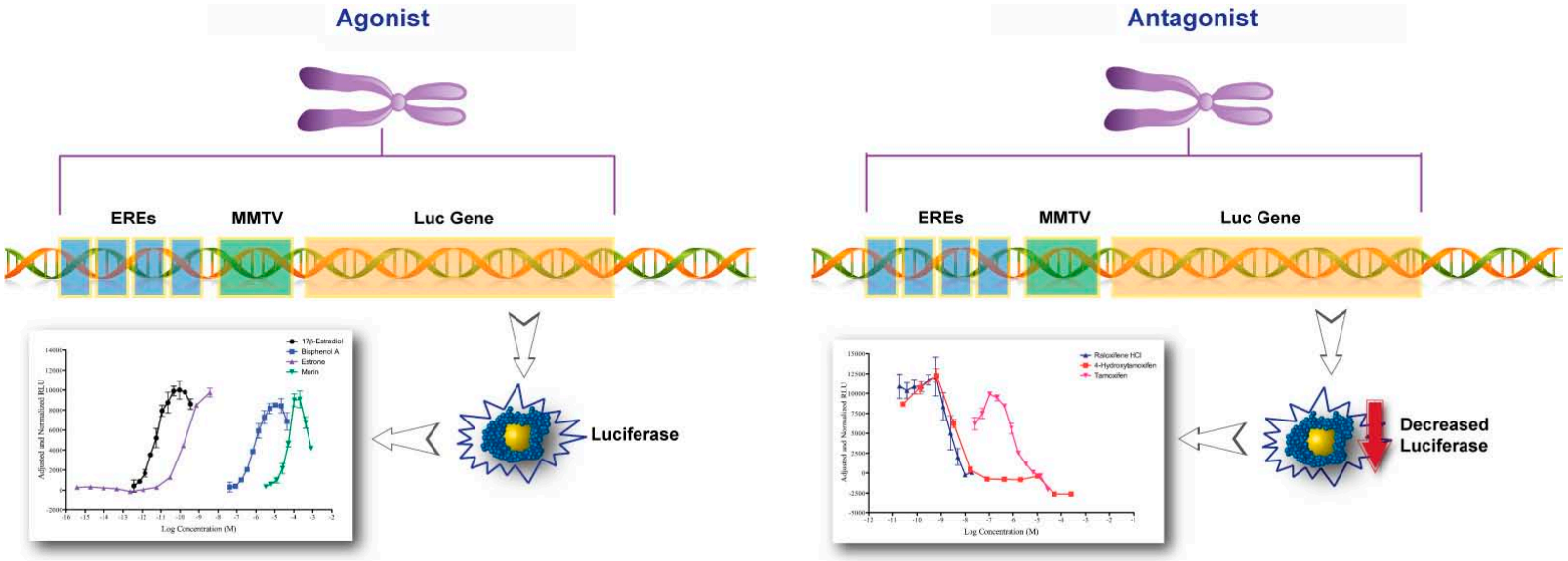


Figure 1. BG1Luc ER TA Agonist and Antagonist Test Methods



Validation Status of BG1Luc ER TA: Test Method Accuracy

- ICCVAM evaluated the BG1Luc ER TA test method for its ability to correctly identify *in vitro* ER agonists and antagonists (**Figures 2** through **4**).
- Test method accuracy was evaluated based on:
  1. The extent to which the result corresponds to the ICCVAM reference classification for each substance (**Figures 2** and **4**)
  2. The extent to which the BG1Luc ER TA test method corresponds to the EPA OPPTS 890.1300/OECD Test Guideline (TG) 455 (EPA 2009; OECD 2009), the currently accepted *in vitro* ER TA test method result (**Figure 3**).

Figure 2. Agonist Accuracy – Comparison of BG1Luc ER TA to ICCVAM Reference Classification

- Thirty-five substances (28 positive, 7 negative) were evaluated to determine the accuracy of the BG1Luc ER TA agonist test method. Agonist accuracy with the ICCVAM reference classification was 97%.
  - Sensitivity = 96% (27/28) — False Negative Rate = 4% (1/28)
  - Specificity = 100% (7/7) — False Positive Rate = 0% (0/7)
  - **Agonist Overall Accuracy = 97% (34/35)**

Figure 3. Agonist Accuracy – Comparison of BG1Luc ER TA to the EPA OPPTS 890.1300/OECD TG 455

- EPA OPPTS 890.1300/OECD TG 455 is the only test guideline published by a U.S. regulatory agency for generating ER TA data. Therefore, accuracy between the BG1Luc ER TA test method and EPA OPPTS 890.1300/OECD TG 455 was also evaluated using the 26 overlapping reference substances for which data are available. Accuracy was 96%.
  - Sensitivity = 95% (21/22) — False Negative Rate = 5% (1/22)
  - Specificity = 100% (4/4) — False Positive Rate = 0% (0/4)
  - **Agonist Overall Accuracy = 96% (25/26)**

Figure 4. Antagonist Accuracy – Comparison of BG1Luc ER TA Test Method to ICCVAM Reference Classification

- Twenty-five substances (3 positive, 22 negative) were used to evaluate the accuracy of the BG1Luc ER TA antagonist test method for correspondence to the ICCVAM reference classification. Antagonist accuracy was 100%.
  - Sensitivity = 100% (3/3) — False Negative Rate = 0% (0/3)
  - Specificity = 100% (22/22) — False Positive Rate = 0% (0/22)
  - **Antagonist Overall Accuracy = 100% (25/26)**
- No comparison could be made with EPA OPPTS 890.1300/OECD TG 455 because it does not include an ER antagonist protocol.

Validation Status of BG1Luc ER TA: Test Method Reliability

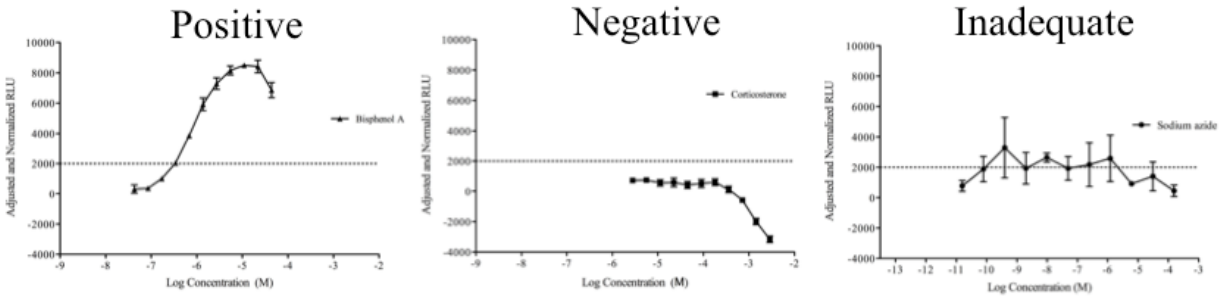
- Intralaboratory reproducibility
  - Agonist testing: 100% agreement within each laboratory for each of the three repeat tests, although the agonist classifications for some of the 12 test substances differed among laboratories.
  - Antagonist testing: 100% agreement within each laboratory for each of the three repeat tests, although the antagonist classifications for some of the 12 test substances differed among laboratories.
- Interlaboratory reproducibility
  - Agonist testing: 100% agreement for the 36 agonist substances that produced a definitive test result in at least two laboratories.
  - Antagonist testing: 93% (38/41) agreement for 41 antagonist substances that produced a definitive test result in at least two laboratories.

Positive and Negative Criteria for the BG1Luc ER TA Agonist and Antagonist Assays

Table 1. Agonist Positive and Negative Decision Criteria

Test Substance Classification	Criteria
Positive	• Test substance has a concentration–response curve consisting of a baseline, a positive slope, and a plateau or peak. In some cases, only two of these characteristics (baseline–slope or slope–peak) may be defined. • The line defining the positive slope must contain at least three points with nonoverlapping error bars (mean ± SD). Points forming the baseline are excluded, but the linear portion of the curve may include the peak or first point of the plateau. • The response amplitude must be at least 20% of the maximal value for the reference estrogen, E2. • If possible, an EC_{50} value should be calculated for each positive substance.
Negative	• The average adjusted relative light unit (RLU) for a given concentration is at or below the mean DMSO control RLU value plus three times the standard deviation of the DMSO RLU.
Inadequate	• Data that cannot be interpreted as valid for showing either the presence or absence of activity because of major qualitative or quantitative limitations are considered inadequate and cannot be used to determine whether the test substance is positive or negative. Substance should be retested.

Figure 5. Examples of Agonist Test Substance Classifications

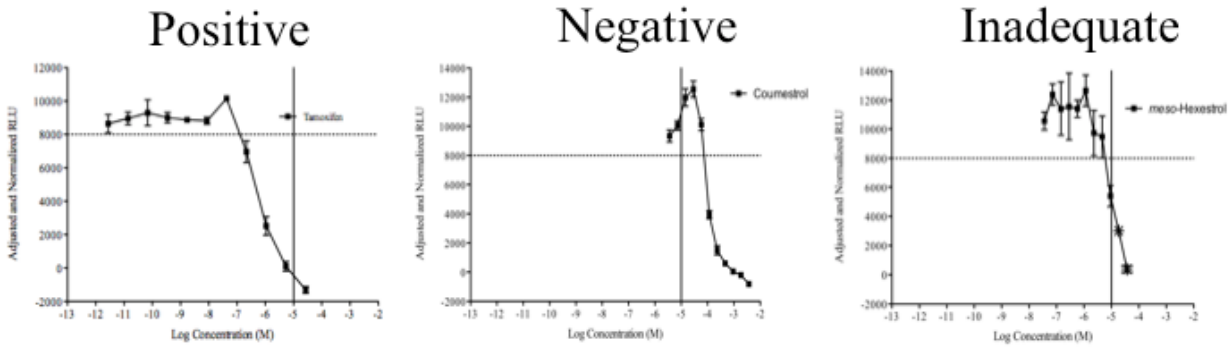


Dashed line indicates 20% of E2 response, 2000 adjusted and normalized RLUs.

Table 2. Antagonist Positive and Negative Decision Criteria

Test Substance Classification	Criteria
Positive	• Test substance has a concentration–response curve consisting of a baseline followed by a negative slope. • The line defining the negative slope must contain at least three points with nonoverlapping error bars (mean ± SD). Points forming the baseline are excluded but the linear portion of the curve may include the first point of the plateau. • The maximum response amplitude should be at least 80% of the maximal value for the reference substance, Ral/E2 (i.e., 8000 RLUs when the maximal response value of the reference substance is adjusted to 10,000 RLUs). As the concentration of the test substance increases, the response curve should decrease below the 80% mark in a dose-dependent manner. • The highest noncytotoxic concentrations of the test substance should be less than or equal to $1x10^{-5}$ M. • If possible, an IC_{50} value should be calculated for each positive substance.
Negative	• All data points are above the ED_{80} value at concentrations less than 1.0×10^{-5} M.
Inadequate	• Data that cannot be interpreted as valid for showing either the presence or absence of activity because of major qualitative or quantitative limitations are considered inadequate and cannot be used to determine whether the test substance is positive or negative. Substance should be retested.

Figure 6. Examples of Antagonist Test Substance Classifications



Dashed line indicates 80% of Ral/E2 response, 8000 adjusted and normalized RLUs.

Solid line indicates 1.00×10^{-5} M. For a response to be considered positive, it must be below the 8000 RLU line, at concentrations less than 1.00×10^{-5} M, and not be cytotoxic.

Asterisks in the *meso*-hexestrol graph indicate concentrations with viability scores of 2 or greater.

meso-Hexestrol is considered inadequate because the only response below 8000 RLU occurs at 1.00×10^{-5} M.

ICCVAM Recommendations: Test Method Usefulness and Limitations

- ICCVAM concludes that the BG1Luc ER TA test method can be used as a screening test to identify substances with *in vitro* ER agonist and antagonist activity.
  - The method can be applied to a wide range of substances, provided that the substances:
    1. Can be dissolved in dimethyl sulfoxide (DMSO)
    2. Do not react with DMSO or the cell culture medium
    3. Are not toxic to the cells at every concentration over the test method's entire range of detection (1.0×10^{-2} to 1.0×10^{-17} M).
- ICCVAM concludes that the accuracy and reliability of this assay is at least equivalent to those of the current ER TA test method included in EPA OPPTS 890.1300/OECD TG 455.

ICCVAM Recommendations: Test Method Protocols

- ICCVAM-recommended protocols for agonist and antagonist testing are available at http://iccvam.niehs.nih.gov/methods/endocrine/ERTA-TMER.htm for all screening activities using the BG1Luc ER TA test method.
- The BG1Luc ER TA test method offers the following advantages over the currently accepted EPA OPPTS 890.1300/OECD TG 455:
  - More detailed and complete test method protocols
  - Validation for testing up to 1 mM per EPA requirements. EPA OPPTS 890.1300/OECD TG 455 is only validated to a limit dose of 10 µM.
  - A more restrictive set of classification criteria for determining a positive response (**Table 1** and **Figure 5**), which will reduce the number of false positive results, resulting in fewer follow-up tests conducted using animals
  - Ability to detect substances with *in vitro* anti-estrogenic activity (**Table 2** and **Figure 6**)
  - Endogenous expression of both hERα and hERβ. The HeLa-9903 cell line used in EPA OPPTS 890.1300/OECD TG 455 was transfected with hERα only.

ICCVAM Recommendations: Future Studies

- To further characterize the BG1Luc ER TA test method, ICCVAM identified the following objectives for additional studies that may be considered by interested parties.

  - More completely characterize the ratio of ERα and ERβ in the BG-1 cell line and the extent to which these receptor subtypes contribute to the overall performance of the BG1Luc ER TA test method.
  - Determine the feasibility of testing volatile substances using CO_2-permeable plastic film or other methods to seal the test plates.
  - Determine if substances that are not soluble in DMSO could be tested in another vehicle that would more adequately solubilize the substance in culture media.
  - As ER antagonists are identified, expand the database of positive substances tested and thereby better characterize the usefulness and limitations of the BG1Luc ER TA test method as a screening test to identify substances with ER antagonist activity.
  - Identify a quantitative method for evaluation of cytotoxicity and account for metabolic activation to expand the utility of this and other ER TA methods.
- ICCVAM encourages users to provide all data that are generated from future studies to ICCVAM so that they may be used to further characterize the usefulness and limitations of the BG1Luc ER TA test method as a screening test to identify substances with in vitro ER agonist or antagonist activity.

ICCVAM Recommendations: Performance Standards

- ICCVAM has developed test method performance standards (ICCVAM 2011a; see Poster 1823) so that modified versions of the BG1Luc ER TA test method that are mechanistically and functionally similar can be effectively and efficiently evaluated for their validity by national and international validation organizations (e.g., ICCVAM, ECVAM, KoCVAM, and JaCVAM) or other organizations.

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BG1Luc ER TA Peer Review Panel Meeting

Independent Scientific Peer Review Panel

Figure Legend

Front Row (Left to Right)	Dr. Sherry Ward (U.S.), Dr. Ellen Mihaich (U.S.), Dr. Warren Casey (U.S.), Dr. John Vandenbergh (U.S.), Dr. William Stokes (U.S.), Dr. William Kelce (U.S.), Dr. Hiroshi Ono (Japan)
Back Row (Left to Right)	Dr. Steven Levine (U.S.), Dr. Grantley Charles (U.S.), Dr. Marc Weimer (Germany), Dr. Christopher Borgert (U.S.), Dr. Charles Eldridge (U.S.), Dr. John Bailer (U.S.), Dr. Daniel Desaulniers (Canada), Dr. James Wittliff (U.S.), Dr. Hyung Kim (Korea)
Not Present for Photo	Dr. Alberto Montovani (Italy), Dr. James Yager (U.S.)

Charge to the Peer Review Panel

- Review the draft background review document (BRD) for completeness and to identify any errors or omissions.

- Evaluate the information in the draft BRD to determine the extent to which each of the applicable criteria for validation and acceptance has been appropriately addressed.
- Consider the draft test method recommendations, and comment on the extent to which they are supported by the information and data in the BRD.

Peer Review Panel Conclusions

- The peer panel agreed that the available data and test method performance support the ICCVAM draft recommendation that the BG1Luc ER TA test method can be used as a screening test to identify substances with *in vitro* ER agonist activity.
  - Nonetheless, the Panel emphasized that, because there has been no clear regulatory guidance on how ER TA test methods will be used in the U.S. EPA Endocrine Disruptor Screening Program, the use of the BG1Luc ER TA test method in the overall strategy of hazard identification or safety assessment of endocrine-disruptive chemicals is unclear.
- The peer panel recommended that the BG1Luc ER TA test method could be considered as a replacement for the currently accepted ER TA assay (EPA OPPTS 890.1300/OECD TG 455).
- The complete BG1Luc ER TA Peer Review Panel Report (ICCVAM 2011b) can be accessed at http://iccvam.niehs.nih.gov/docs/endo_docs/EDPRPRept2011.pdf
- The Test Method Evaluation Report (ICCVAM 2011a) has been forwarded to member agencies for review and comment.

International Acceptance of the BG1Luc ER TA Test Method

After the Panel review, a draft OECD Test Guideline was developed based on the BG1Luc ER TA performance standards and sent to the Organisation for Economic Co-operation and Development for review.

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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.

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