Appendix B

Conduct and Analysis of Edging Effects Experiments

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1.0 Range Finder Testing: Impact of the Revised Plate Design

Some studies have suggested that including all wells could bias responses because of so-called "edging effects" (Nagy 2002, Oliver 1989) resulting from differences in vapor pressure between the outer wells (n = 36) as compared to the inner wells (n = 60) of a 96-well plate. For this reason, during the protocol standardization, a plate layout using only the 60 inner wells of the 96-well plate was used.

However, use of 60 out of 96 wells on each 96-well plate is inefficient, allowing only 62.5% of all available wells to be used.

In order to increase efficiency, a series of experiments were conducted to test the impact of a revised plate layout using all 96-wells in the 96-well plate was conducted. The experiments concentrated on range finder testing and the resulting concentrations that would be selected for comprehensive testing.

The evaluation of edging effects for the revised range finder plate design using all 96-wells addressed the following questions:

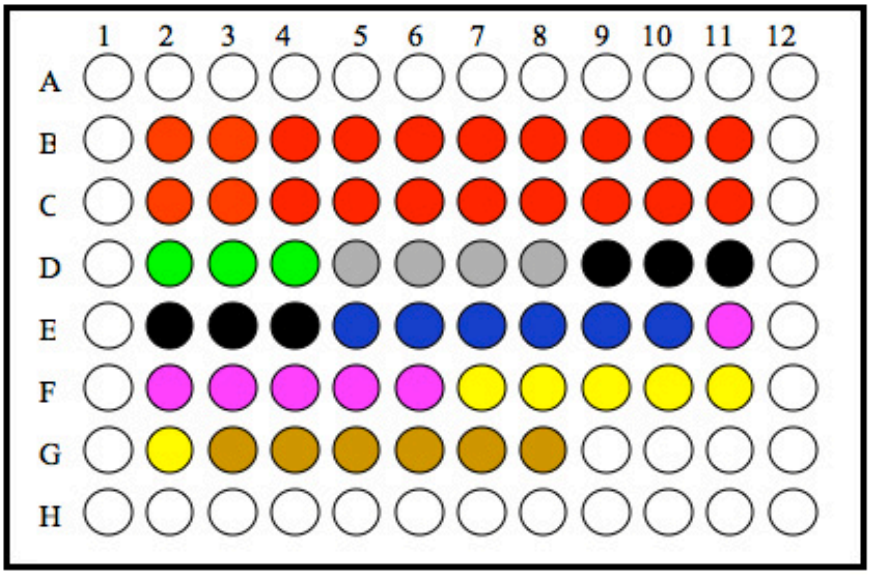
- Are there significant differences in observed responses (as measured in relative light units [RLUs] recorded from a luminometer) between outside and inside wells (edging effects) using the revised plate design?
- If edging effects are observed in range finder testing, do they have a significant impact on the selection of concentrations for comprehensive testing?

2.0 Evaluation of Possible Edging Effects

The respective agonist and antagonist range finder plate layouts using inner wells only are provided in **Figures B-1** and **B-2** and the respective agonist and antagonist revised range finder plate layouts using all wells are provided in **Figures B-3** and **B-4**.

To evaluate the revised plate layouts for possible edging effects, observed responses (i.e., RLUs) between outside and inside wells were compared for ten plates (**Table 1**) using the BG1LUC4E2 ER TA assay agonist protocol to test seven point logarithmic (log) serial dilutions of bisphenol A (BPA, 100 µg/mL – 1 $x10^{-4}$ µg/mL) in the revised plate layout and seven plates (**Table 2**) using the antagonist protocol to test seven point log serial dilutions of tamoxifen (50 µg/mL – 5 $x10^{-5}$ µg/mL) in the revised plate layout. Serial dilutions were run in plate columns 1–12 at descending concentrations in rows A-G. A comparison of RLUs was made between column 1 (left outside wells) and column 2 (adjacent left inside wells), and column 12 (right outside wells) and column 11 (adjacent right insides wells), and using rows B-G (row A was excluded because it is the top outside row on the plate). A total of 204 pairs were evaluated with a Z statistic ($Z \geq 1.96$, 95% confidence interval) using a Sign Test to determine significant differences between outer and inner wells (Zar 1984). Analysis of paired values in columns 1 and 2, and 11 and 12 resulted in Z statistics of 5.67 and 2.87 respectively, indicating statistically significant differences in observed RLUs between outer and inner wells.

Figure B-1 Protocol Standardization Agonist Range Finder Plate Layout



Duplicate 10 Point Reference Standard

Methoxychlor Positive Control)

DMSO (Solvent Control)

Test Substance #1

Test Substance #2

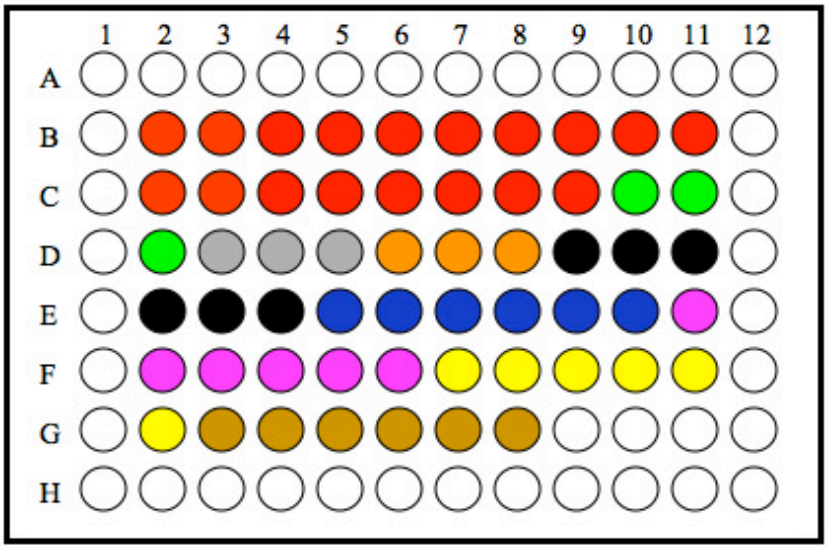
Test Substance #3

Test Substance #4

Test Substance #5

Media only wells, not used for assay

Figure B-2 Protocol Standardization Antagonist Range Finder Plate Layout



Duplicate 9 point Ral/E2 Reference Standard

Flavone Positive Control

DMSO (Solvent Control)

E2 Control

Test Substance #1

Test Substance #2

Test Substance #3

Test Substance #4

Test Substance #5

Media only wells, not used for assay

Figure B-3 Revised Agonist Range Finder Plate Layout

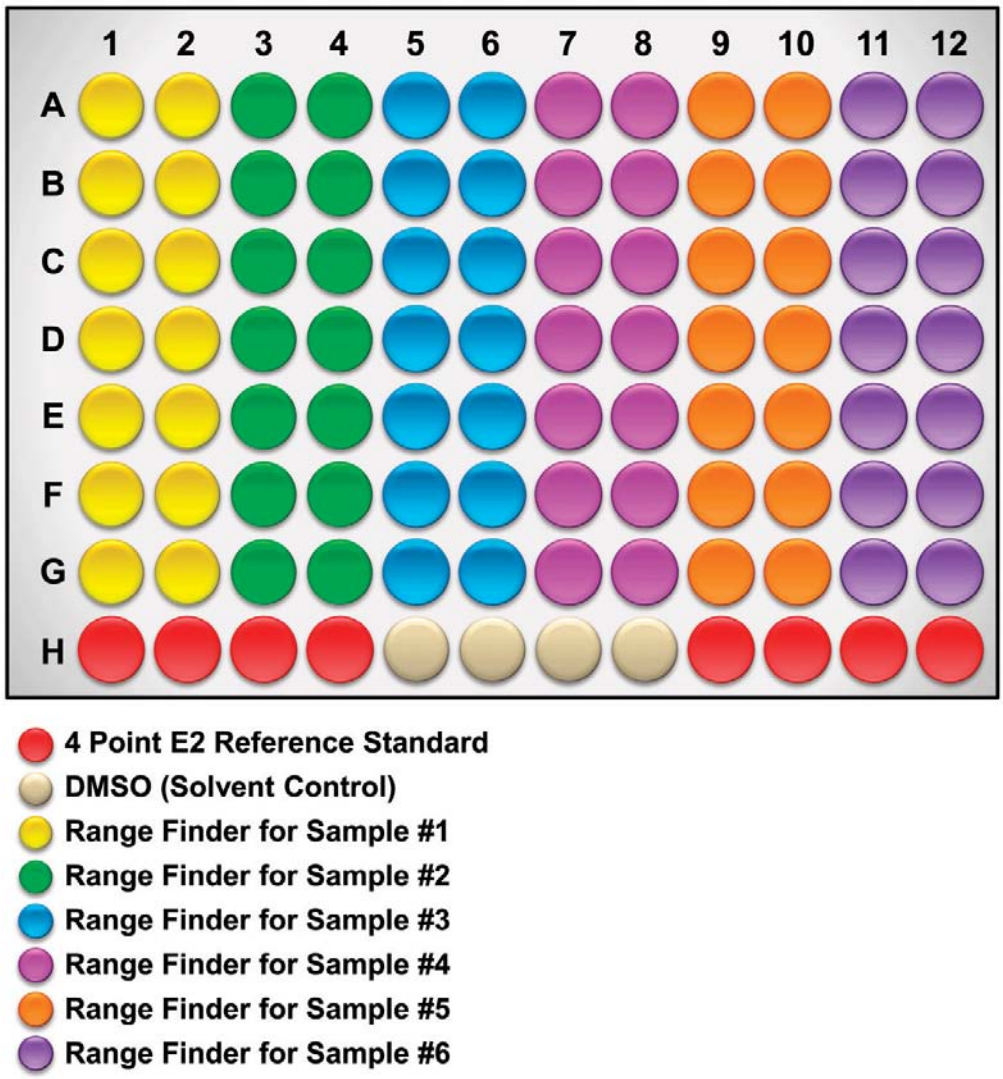


Figure B-4 Revised Antagonist Range Finder Plate Layout

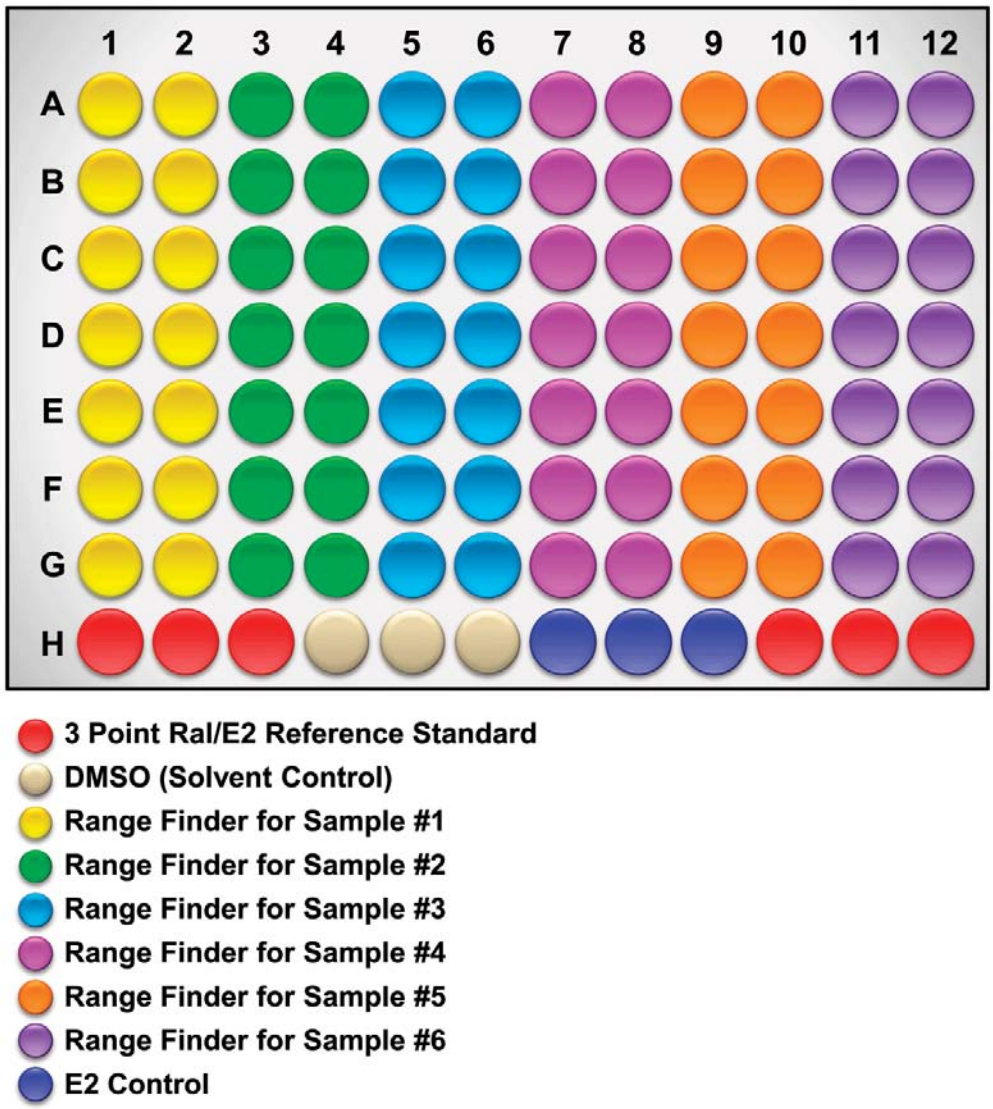


Table B-1 Revised Agonist Range Finder Plates Tested

Experiment I.D.	Date	Induction[1]
N2-All-1	13-Jun-07	4.9
N2-All-2	13-Jun-07	4.6
N2-R-All-3	15-Jun-07	4.8
N2-R-All-4	15-Jun-07	4.5
ALL-BPA-1	29-Jun-07	3.0
ALL-BPA-2	29-Jun-07	4.3
ALL-BPA-3	29-Jun-07	5.9
ALL BPA-1	05-Jul-07	5.5
ALL BPA-2	05-Jul-07	5.3
ALL-BPA-3	05-Jul-07	6.6

[1] Induction for range finder plates is measured by dividing the averaged highest E2 reference standard RLU value by the averaged DMSO control RLU value.

Table B-2 Revised Antagonist Range Finder Plates Tested

Experiment I.D.	Date	Reduction[1]
Tam BM 1	19-Jul-07	3.7
Tam BM 2	19-Jul-07	4.2
Tam John 1	19-Jul-07	4.2
Tam John 2	19-Jul-07	4.6
Tam All 1	03-Aug-07	4.5
Tam All 2	03-Aug-07	4.9
Tam All 3	03-Aug-07	4.8

[1] Reduction for range finder plates is measured by dividing the averaged highest Ral/E2 reference standard RLU value by the averaged DMSO control RLU value.

2.1 Impact of Edging Effects on Concentration Selection for Comprehensive Testing

Range finder testing in the BG1LUC4E2 ER TA assay is used to select the appropriate concentration range for comprehensive testing. For substances with apparent estrogenic activity based on results in the range finder testing, the starting concentration that is selected for comprehensive testing (and subsequently diluted in 11-point double serial dilutions) is one log dilution higher than the concentration giving the highest RLU value. **Figure B-5** presents an example of edging effects in an agonist range finder test of BPA (plate N2-R-All-3 in **Table B-1)** in which the RLUs from plate column 12 (outside right column) are significantly different. This difference results in a concentration-response curve that is clearly lower in magnitude than the remaining curves. However, the shape of all 12 concentration-

response curves are similar and importantly, the concentration giving the highest RLU value (10 µg/mL) is identical in all columns.

For substances with apparent anti-estrogenic activity based on results in the range finder testing, the starting concentration that is selected for comprehensive testing (and subsequently diluted in 11-point double serial dilutions) is one log dilution higher than the concentration giving the lowest RLU value or the maximum soluble concentration. **Figure B-6** presents an example of edging effects in an antagonist range finder test of tamoxifen (plate Tam BM 1 in **Table B-2)** in which the RLUs from plate column one (outside left column) are significantly different. Again, although a concentration-response curve that is lower in magnitude results from this difference, the shape of all 12 concentration curves are essentially the same and the concentration giving the lowest RLU value (50 µg/mL) is the same in all columns. Therefore, the same concentration would be selected for comprehensive testing.

These examples demonstrate that although there are statistical differences between RLUs in the outer and inner wells, these differences do not impact selection of the appropriate starting concentration for comprehensive testing for either the agonist or antagonist protocols.

Figure B-5 Agonist Range Finder Testing of Bisphenol A

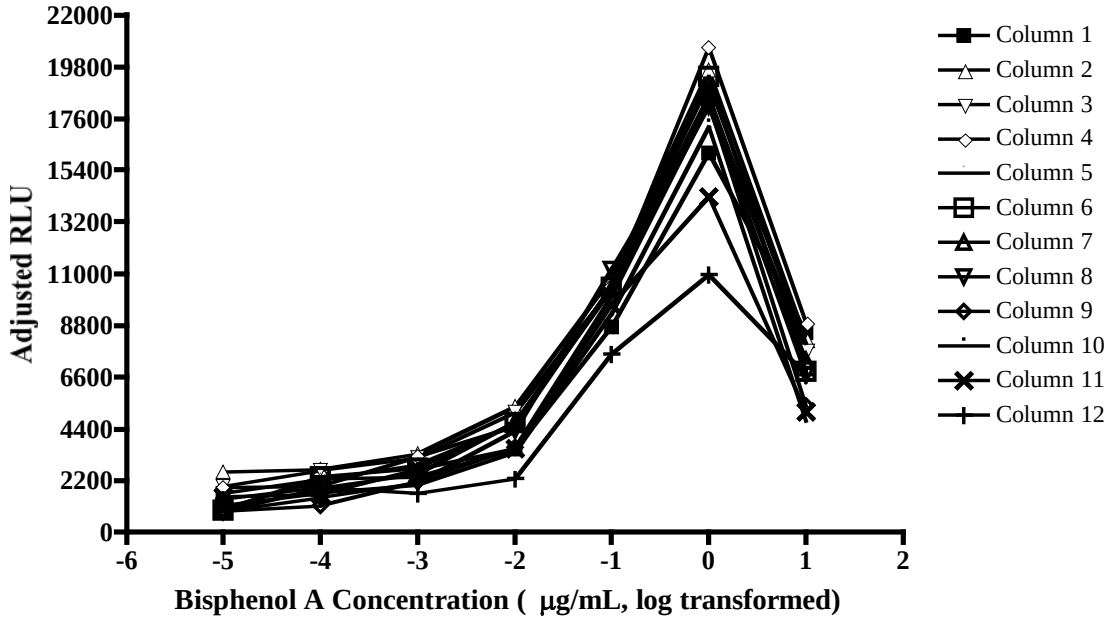
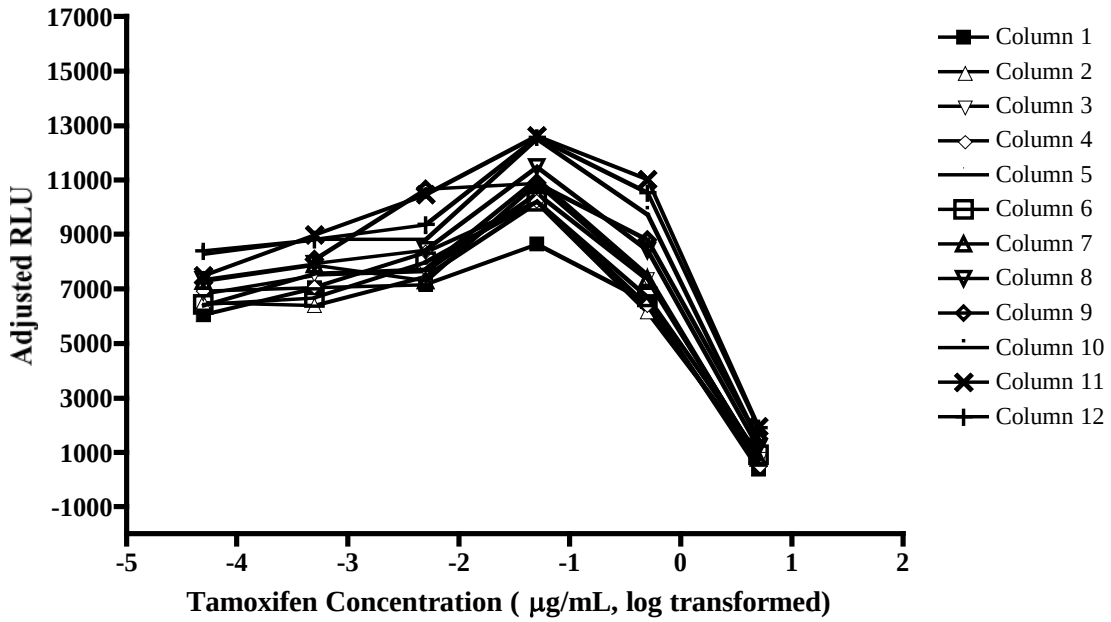


Figure B-6 Antagonist Range Finder Testing of Tamoxifen



2.2 Impact of Edging Effects on Comprehensive Testing Results

To maximize throughput of testing in the BG1LUC4E2 ER TA assay without sacrificing statistical power in the study design, the plate layouts for agonist and antagonist comprehensive testing have also been revised from the original layouts presented in **Figures B-7** and **B-8** to use all 96 wells. This will allow the testing of 11-point double serial dilutions of two substances in triplicate (see **Figures B-9** and **B-10**), instead of only one substance when using the original plate layout. The revised plate layout for comprehensive testing uses row A (outside wells) for one of the three replicates of a test substance concentration range. To evaluate the effect of using these outer wells on comprehensive test results in the agonist protocol (i.e., the calculated EC_{50}[1] values), data from ten plates using the BG1LUC4E2 ER TA assay agonist protocol to test logarithmic serial dilutions of bisphenol A BPA, in the revised plate layout described in **Section 2.1** were used to calculate EC_{50} values for BPA. Averaged EC_{50} values derived from replicates using outside wells (triplicate serial dilutions using columns 1, 2 and 3, and columns 10, 11 and 12) were compared to averaged EC_{50} values using inside wells (triplicate serial dilutions using columns 4, 5 and 6, and columns 7, 8 and 9). The comparison of EC_{50} values was conducted using the Friedman Test, a nonparametric test that compares matched groups by ranking group values and conducting a two-way analysis of variance (Hollander 1973). Based on this analysis no significant difference was observed ($p > 0.05$) between EC_{50} values derived from replicates using outside wells and those derived from inside wells only.

These results suggest that, although statistical differences were noted between RLUs from outer and inner wells, these differences did not result in biologically significant differences in comprehensive testing results for the agonist protocol (i.e., EC_{50} values). Based on the similarities in results described in **Sections 2.1** and **2.2**, biologically significant differences in comprehensive testing results for the antagonist protocol (i.e., IC_{50}[2] values) are not anticipated. However, additional analyses will be conducted

[1] EC_{50} = half maximal effective concentration

[2] IC_{50} = concentration of the test substance inhibiting the reference estrogen response by 50%

during the international validation of the BG1LUC4E2 ER TA assay to further characterize any impact of edging effects.

Figure B-7 Protocol Standardization Agonist Comprehensive Test Plate

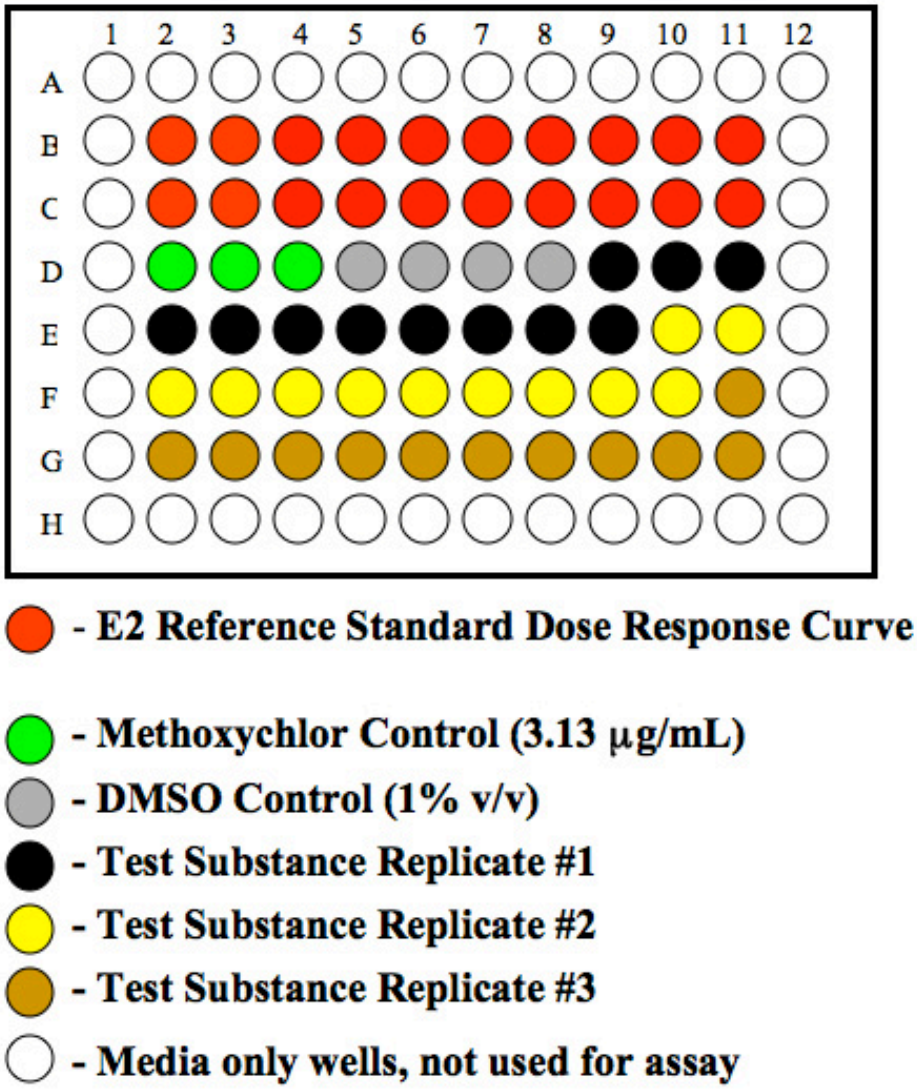


Figure B-8 Protocol Standardization Antagonist Comprehensive Test Plate Layout

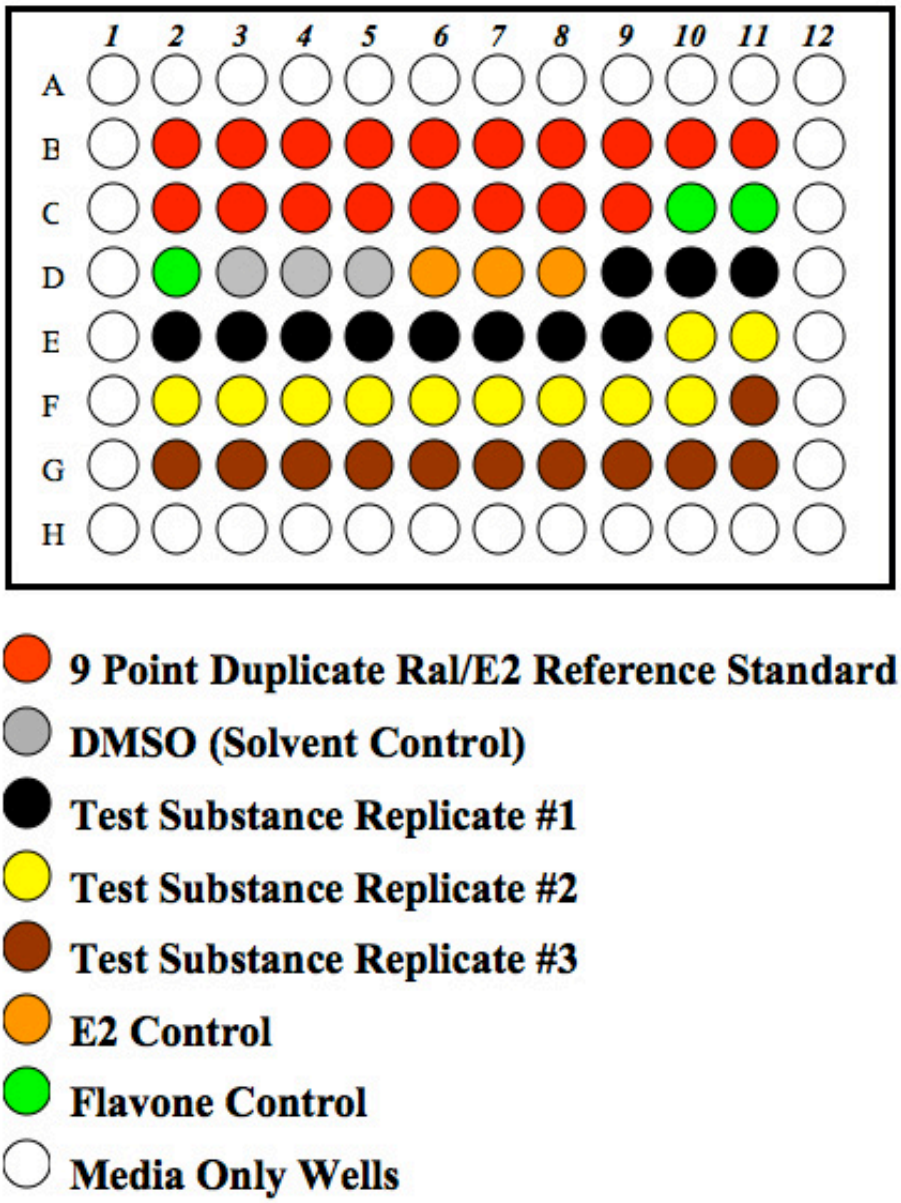


Figure B-9 Revised Agonist Comprehensive Test Plate Layout

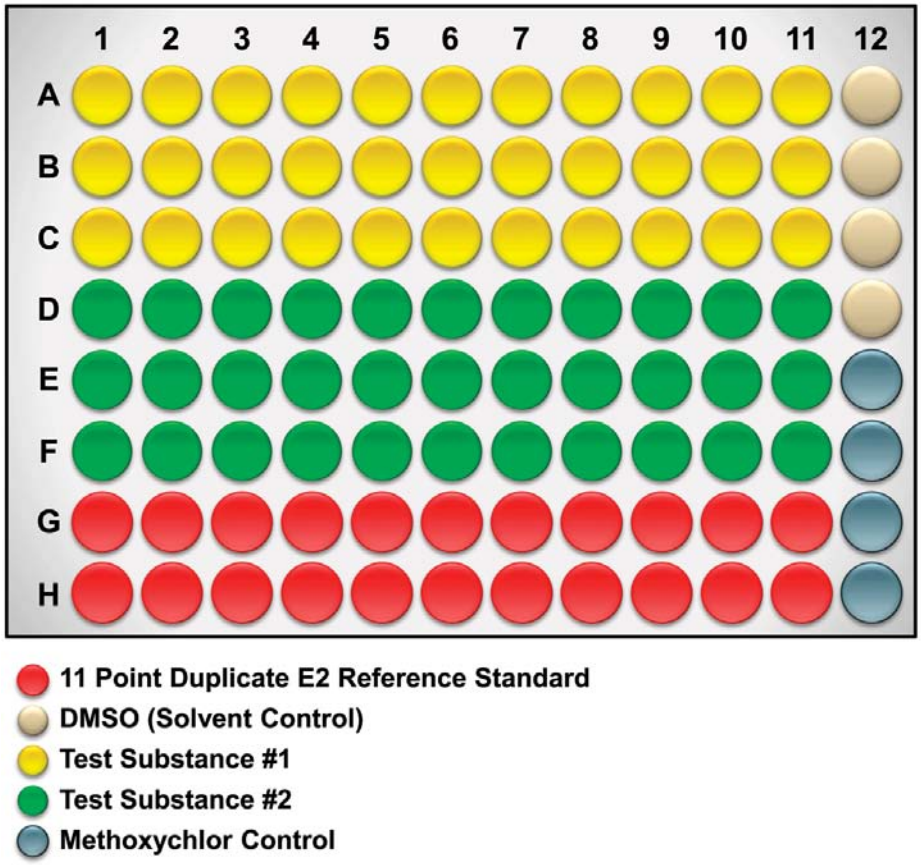
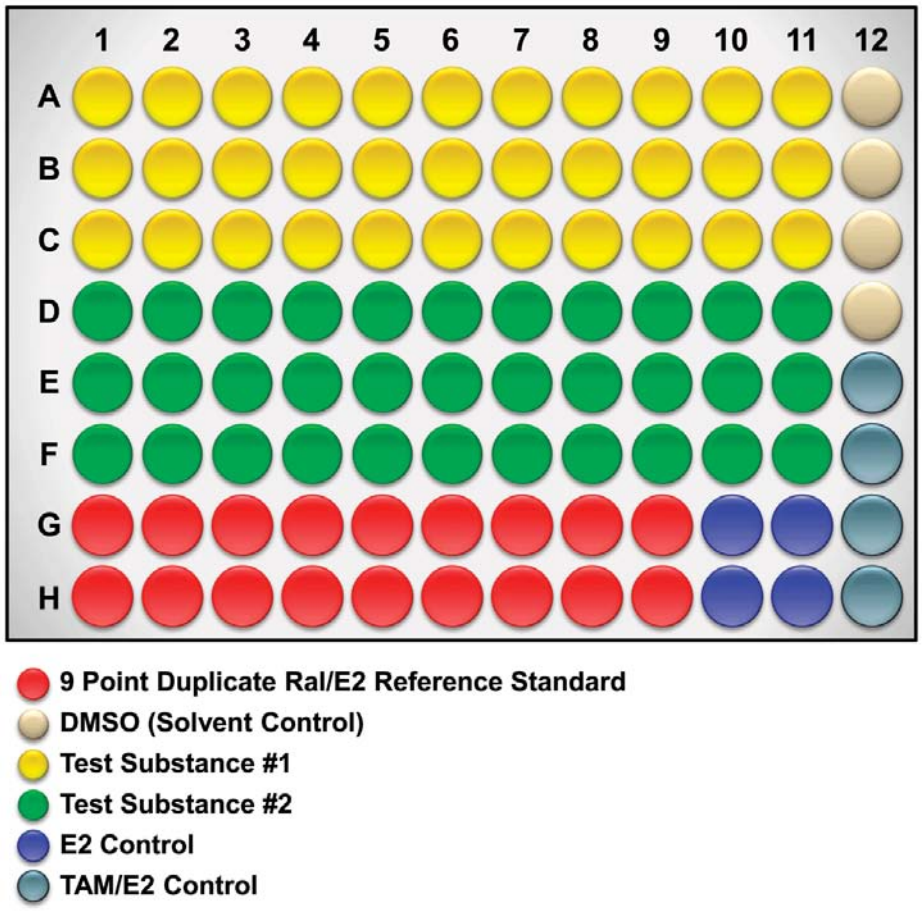


Figure B-10 Revised Antagonist Comprehensive Test Plate Layout



3.0 Plate Design Amendments to BG1LUC4E2 ER TA Assay Protocols

Following the review and discussion of the above results during their 25 July 2007 teleconference, the BG1LUC4E2 ER TA Assay Validation Study Management Team agreed to modify the BG1LUC4E2 ER TA Assay validation study test plate designs to use all wells of 96 well agonist and antagonist test plates for range finder and comprehensive testing and a study amendment outlining the plate design modifications was written (see **Study Amendment II** in **Appendix I**).

4.0 References

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Oliver MH, Harrison NK, Bishop JE, Cole PJ, Laurent GJ. 1989. A rapid and convenient assay for counting cells cultured in microwell plates: application for assessment of growth factors. J Cell Sci 92:513-518.

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