MICROPLATE EDGE EFFECTS IN RANGE FINDING STUDIES USING THE LUMI-CELL® ER BIOASSAY: INCREASING ASSAY THROUGHPUT

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Abstract
Concern over the detrimental effects of endocrine disruptor chemicals (EDCs) has prompted the need for an effective high throughput system for detection of EDCs. Typically, the outer wells of a 96-well plate are not used for bioassay analysis due to edge effects, which have been found to reduce measured activity. This results in fewer available assay wells, in effect decreasing analysis throughput by 38%. Ring analysis of the 96-well plate and range finding studies with Bisphenol A and Bisphenol B in the LUMI-CELL® ER bioassay revealed no significant difference (after t-test analysis) between using the inner 60 or all 96 wells of a 96-well microplate. Utilizing all 96 wells in the assay will increase throughput of the assay by 38% over that of typical cell bioassays that use only the 60 inner wells of the plate.

Introduction
The strong association between the exposure and bioaccumulation of endocrine disruptor chemicals (EDCs) and their adverse effects on human and wild life populations has given rise to concerns worldwide over the past several years[1,2]. Some examples of the effects of EDCs are: decreased reproductive success and feminization of males in several wildlife species; increased hypospadias along with reductions in sperm counts in men; increase in the incidence of human breast and prostate cancers; and endometriosis[3-5]. In light of these detrimental effects of environmental exposure to EDCs, there is an obvious need to increase throughput of screening systems for detection of EDCs.

In typical microplate cell bioassays, the outer wells of a 96-well plate are not used because of "edge" effects. These edge effects were indicated to result in decreased sensitivity and increased variability in sample readings as a result of a lack of constant humidity and temperature resulting in uneven evaporation[7]. While there are products produced, such as covered plates and incubators with faster temperature and humidity recovery times, to reduce edge effects, there are very few published studies, which actually document edging effects. Lack of inclusion of the 36 outer wells of a 96-well plate in bioassays, results in a reduction in sample throughput, effectively reducing assay throughput by 38% of total capacity. In this report we conducted 2 studies to examine the variability of the "outer" wells compared to that of the "inner" 60 wells. Bisphenol A (BPA) and Bisphenol B (BPB) from the ICCVAM sponsored protocol standardization study of the LUMI-CELL® ER bioassay were used to determine variability in actual range finding studies using both inner and outer wells.

Methods and Materials
Compound Preparation and Analysis Procedure: In the first study a dosing concentration of 2.5×10^{-5} µg/ml 17β-estradiol (E2) or 3.13 µg/ml Methoxychlor was plated in all 96 wells. An analysis of the outer ring and all successive inner rings were then compared to the inner 60 wells. The data from two plates of E2 and one plate of Methoxychlor were averaged and the data presented here. In the second study, a 10 mg/ml solution of each compound (BPA and BPB) was prepared in DMSO. A range finding assay was preformed on each compound using six log dilutions. A group comparison t-Test was preformed to determine any significant differences.

LUMI-CELL® ER Bioassay. The BG1Luc4E2 cell line was constructed as previously described by Rogers and Denison (2000). Briefly, BG1 cells were stably transfected with an estrogen-responsive luciferase reporter gene plasmid (pGudLuc7ere) and selected for G418 resistance[5]. For analysis, the BG1Luc4E2 cell clone resulting from this selection was grown in RPMI 1640 medium. The cells were transferred into flasks containing phenol red-free DMEM media supplemented with charcoal dextran stripped FBS and incubated for four days before harvesting for BG1Luc4E2 bioassay plating. The cells were then plated in white clear-bottomed Corning 96 well

microplates and incubated at 37°C for 24-48 hours prior to dosing. The media solution in each well was removed and two hundred microliters of phenol red-free DMEM supplemented with charcoal dextran stripped FBS containing the indicated concentration of the desired chemical to be tested was added to each well. The plate was then incubated for 20 hours 37°C before analysis of luciferase activity.

Measurement of Luciferease Activity. After lysing the cells (Promega lysis buffer), the luciferase activity was measured in a Berthold Orion Microplate Luminometer, with automatic injection of 50 micro liters of luciferase enzyme reagent (Promega) into each well. The relative light units (RLUs) measured were compared to that induced by the E2 standard after subtraction of the background activity.

Results and Discussion

In the first study, a ring analysis was conducted of the 96-well plates. The entire plate was dosed with one concentration of E2 ($2.5x10^{-5}$ µg/ml (91.8 pM)) or Methoxychlor (3.13 µg/ml (905.4 µM)). The data from two plates of E2 and one plate of Methoxychlor were averaged and the data presented here. The outer ring (Ring 1) and each successive inner ring were compared to the normally used inner 60 wells (Figure 1). Luciferase activity, of lysed cells in wells in Ring 1, was 7% lower than that in the inner 60 wells, while activity in cell lysates in wells in Ring 2 were 3% lower. Cell lysates in wells in Rings 3 and 4 were each 1% higher than that of lysates in the inner 60 wells. Each of the top and bottom rows, as well as the left and right columns were also compared to the inner 60 wells. The outer wells were compared by row and column (Table 1B) and ranged from 4% - 10% lower than the inner 60 wells. None of the ring analysis or any of the outer rows and columns demonstrated any significant difference from the inner 60 wells when analyzed by t-test (Tables 1A and 1B). While our results demonstrate an overall decrease in activity in cell lysates in the outer wells, the activities were much higher and less variable than previously reported, indicating the potential of using these wells for LUMI-CELL® ER screening or range finding studies.

Figure 1. Ring analysis of 96-well plate using the LUMI-CELL® ER bioassay. Each ring of data (Ring 1, 2, 3, and 4) was compared to the inner 60 wells contained within the dashed lines below.

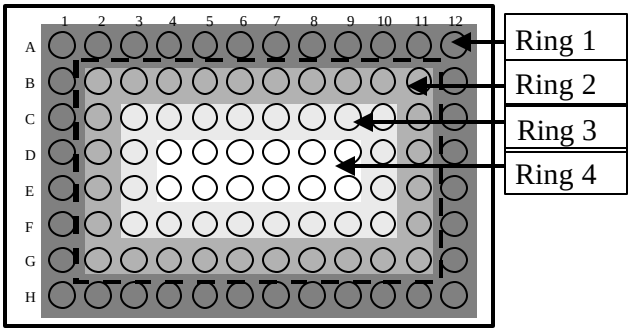


Table 1A and 1B. Ring and outer well analysis of RLU Data from E2 and Methoxychlor on 96-well plate. In Table 1A, the mean from each ring was compared to the mean of the inner 60 wells. In Table 1B, mean from each outer row and column was compared to the mean of the inner 60 wells.

A

	Mean	SD	$t_{0.95}$
Ring 1	93%	5%	0.92
Ring 2	97%	1%	1.04
Ring 3	101%	1%	-0.63
Ring 4	101%	1%	-0.49

B

	Mean	SD	$t_{0.95}$
top row	94%	7%	0.53
bottom row	90%	6%	0.84
lef column	91%	3%	1.16
right column	96%	10%	0.55

In the second study, a range finding study was conducted to determine the edge effects on actual samples. Solutions of BPA and BPB (10 mg/ml each) were prepared in DMSO. A range finding assay was preformed on

each compound using seven log dilutions. Each compound was then run vertically in the 96-well plate using the outer wells in triplicate in two locations on the plate (Figure 2A). These same compounds were then run horizontally within the normal inner 60 wells used in most assays (Figure 2B). The results from each plate were compared to determine the variability of the two dosing methods. For both BPA and BPB, there was no significant difference ($t_{0.95} \leq 2.09$ for all concentrations of BPA and $t_{0.95} \leq 3.16$ for all concentrations of BPB) in the response of the LUMI-CELL® ER bioassay when the compounds were plated vertically using the outer wells and dosed horizontally using just the inner 60 wells (Figure 3 and 4). The far left column in Figure 2A, testing BPA, demonstrates no significant difference ($t_{0.95} \leq 1.00$ for all concentrations tested) from any of the other inner columns dosed. The far right column in Figure 2A, testing BPB, demonstrates no significant difference ($t_{0.95} \leq 2.08$ for all concentrations tested) from any of the other analysis. There appears to be no significant difference in the data from the current study and the data submitted to ICCVAM for the LUMI-CELL® ER Protocol Standardization Study, which was dosed as in Figure 2B and seen in Figures 3 and 4. The highest concentration for both BPA and BPB were cytotoxic and therefore not included in the graphs.

Figure 2. Range finder analysis for BPA and BPB using the LUMI-CELL® ER bioassay. Figure 2A depicts the plate set up for analysis using all 96 wells. Figure 2B depicts the plate setup for normal testing using only the inner 60 wells.

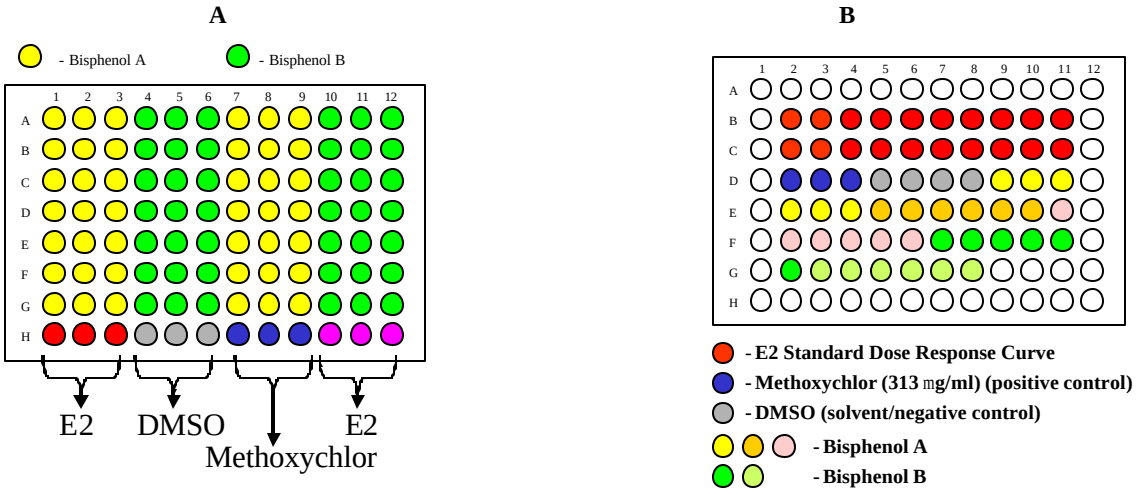


Figure 3. Range finder analysis of BPA analyzed by both plate setups depicted in Figure 2A (normal) and 2B (96). The BPA - ILS line represents the data for BPA presented to ICCVAM during the protocol standardization study for the LUMI-CELL® ER bioassay.

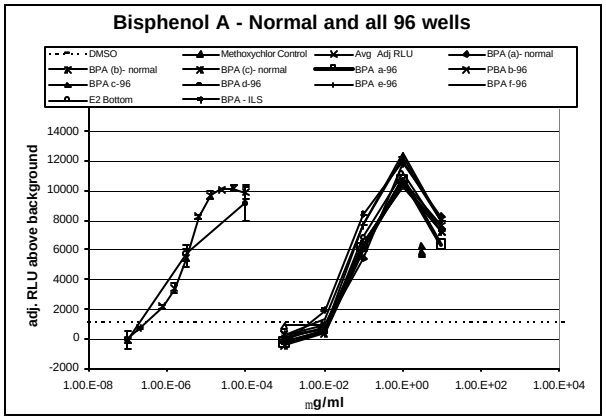
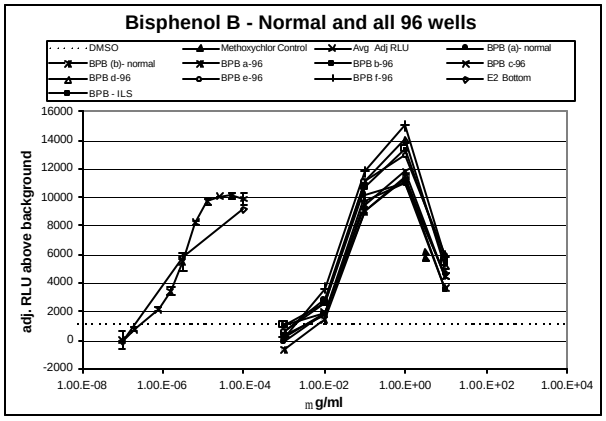


Figure 4. Range finder analysis of BPB analyzed by both plate setups depicted in Figure 2A (normal) and 2B (96). The BPB - ILS represents the data for BPB presented to ICCVAM during the protocol standardization study for the LUMI-CELL® ER bioassay.



The data presented here using the ring analysis and range-finder analysis, demonstrate the responsiveness and low variability of the outer wells of a 96-well plate in the LUMI-CELL® ER bioassay. After t-test analysis of all data there was no significant difference in any of the data analysis. This data further supports the use of these wells in screening or range finding studies, which will result in a 38% increase in sample throughput for the LUMI-CELL® ER bioassay. Studies are currently in progress to determine whether similar results are obtained with other cell based bioassays (i.e. XDSCALUX bioassays for dioxin-like chemicals and androgenic chemicals).

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