ROBUSTNESS OF THE CALUX BIOASSAY: STATISTICAL ANALYSIS OF THE BETWEEN-WELL VARIABILITY FOR THE H1L6.1C3 MOUSE HEPATOMA CELL LINE

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Abstract

The variation of the CALUX response between different wells on a 96-well plate was investigated for the H1L6.1c3 mouse hepatoma cell line. Analysis of 6 plates, spiked with 1.5 pg/µL of a 2, 3, 7, 8 -TCDD standard solution, on 3 different days indicated significant differences in RLU values between the wells. The mean RLU values were significantly lower ($p = 1.43E-12$) in the outer circle of the plate (i.e. rows A and H, and columns 1 and 12) and there were significant differences found between both columns and rows in a two-way ANOVA ($p < 0.001$). Even after rejection of the outer wells of the plate, significant differences were still found between the rows, columns (both p-values < 0.001) and circles ($p = 0.002$).
Therefore, it is important to pay attention to the spatial distribution of the samples, standards and blanks on the plate and to take into account the between-well variability on a 96-well plate.

Introduction

The CALUX (Chemical Activated LUciferase gene eXpression) assay is a reporter gene mammalian cell bioassay that is nowadays often used as a rapid and inexpensive screening and semi-quantitative method for the analysis of PCDD/Fs in several matrices, such as milk[1], blood plasma[2], sediments[3] and marine biological matrices[4].
The recombinant cells used in the CALUX bioassay contain a stably transfected AhR-responsive firefly luciferase reporter gene, which responds by the induction of luciferase. The measured luminescence is converted into a bioassay toxic equivalency value (CALUX-TEQ) by the comparison of the response for a given sample to a dose-response curve obtained with 2, 3, 7, 8-TCDD standards[5, 6]. Generally, a four parameters Hill-plot is used to fit a sigmoid curve through the standard solutions and 3 quality control solutions are added in duplicate to the cell plate to calculate a coefficient of variation (CV).
Since many samples contain low concentrations of dioxin-like compounds and are often available in small amounts (e.g. in biomonitoring studies), the extracts are sometimes dosed on the cell plate in a single well. Therefore, it is important to have an estimate of the variability.
This paper presents between-well variability of the cell response for the H1L6.1c3 mouse hepatoma cell line, both within and between 96-well plates.

Materials and Methods

Calux assay
The cell line used in the bioassay was a modified cell line H1L6.1c3, which was obtained from M. Denison, University of California-Davis, USA.
Cell treatment and measurement were based on the protocols described by Windal et al[4]. and the XDS method 4435[7]. The cells were grown in a 75 cm² culture flask containing 20 µL RPMI 1640 supplemented with 8% FCS and 1% penicillin/streptomycin (Gibco, UK). After trypsinizing, the cells were counted and diluted to a concentration of ± 70E4 cells per mL. For a reliable statistical interpretation, six 96-well plates were analyzed

on three different days. Every well on the plate was seeded with 200 µL cell suspension. After 24 h incubation (37°C, 5% CO_2), 188 µL of a 1.5 pg/µL quality control 2,3,7,8 TCDD standard solution (Campro Scientific, Germany) in DMSO was added to every well on the plate. After 24 h incubation, the medium was removed and the wells were rinsed with 75 µL PBS buffer pH 7.4 (Gibco, UK). Then the plate was visually inspected under the microscope in order to determine possible cell death, 30 µL lysis reagent (Promega, USA) was added and the plate was shaken for 5 minutes. After a 10 minute incubation period in the luminometer (Glomax, Promega, USA), 50 µL luciferine reagent was injected and the light output was given in RLUs (integration time 5 s, lag time 5.6 s). The luminometer was configured in a way that the plate had to be read in row-wise manner, starting in A1 and ending with H12 (Figure 1).

Statistical data treatment

Database management and statistical analysis were performed using SigmaStat 3.10. The raw data were first tested for outliers using the inter-quartile range method and then normalized to account for differences in cell concentration and incubation time. One way and two way ANOVA tests and Holm-Sidak and Ducan's multiple range post hoc tests were used to indicate significantly different mean values.

Results and Discussion

The raw data of the 6 plates are given in Table 1. From this table, it is clear that the overall values were consistently higher for the second plate analyzed on the same day.

	Day 1 plate 1	Day 1 plate 2	Day 2 plate 1	Day 2 plate 2	Day 3 plate 1	Day 3 plate 2
Smallest value	156 536	221 214	141 027	166 104	164 492	215 921
1st quartile	189 991	259 563	165 748	200 560	208 637	245 046
Median	203 693	284 328	180 697	212 773	231 345	265 645
3rd quartile	215 872	298 814	192 921	227 986	244 088	288 602
Highest value	237 695	338 948	214 539	249 819	271 279	335 988

Table 1: statistics of the 6 plates

For every well, the mean normalized RLU and coefficient of variation (CV) of the 6 plates were calculated. As can be seen in Table 2 and Figure 1, there are obvious differences between the wells. Rows A, G and H, and column 1 and 2 have a lower response, while column B and C have the highest responses. Coefficients of variation were also higher for the outer wells (mean of all wells in Table 2 was 6.6%). When considering the between-well CV for the 6 plates separately, the CVs ranged between 8.0 and 10.8%.

	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**
A	11.8	10.4	10.7	6.2	6.0	5.9	5.6	6.7	6.9	4.4	5.6	8.3
B	8.7	7.6	5.1	7.0	2.1	6.7	2.2	4.3	4.7	7.4	5.8	4.8
C	9.0	7.5	5.9	5.6	4.4	2.3	6.7	4.1	2.8	7.5	6.0	9.1
D	11.6	3.2	7.0	7.9	4.8	3.7	4.4	7.1	6.6	6.9	7.9	7.8
E	12.9	4.8	6.6	3.4	5.1	4.5	5.2	4.8	7.1	6.0	6.3	9,7
F	7.8	10.1	9.1	6.1	2.3	2.5	4.1	6.1	4.8	6.4	8.1	11.3
G	8.2	6.1	5.2	3.6	4.4	5.6	4.1	9.0	5.7	8.5	9.0	13.7
H	10.1	9.0	8.3	6.9	7.2	5.7	7.4	6.5	6.1	9.8	10.5	9.1

Table 2: CV for all 96-wells, averaged over 6 plates

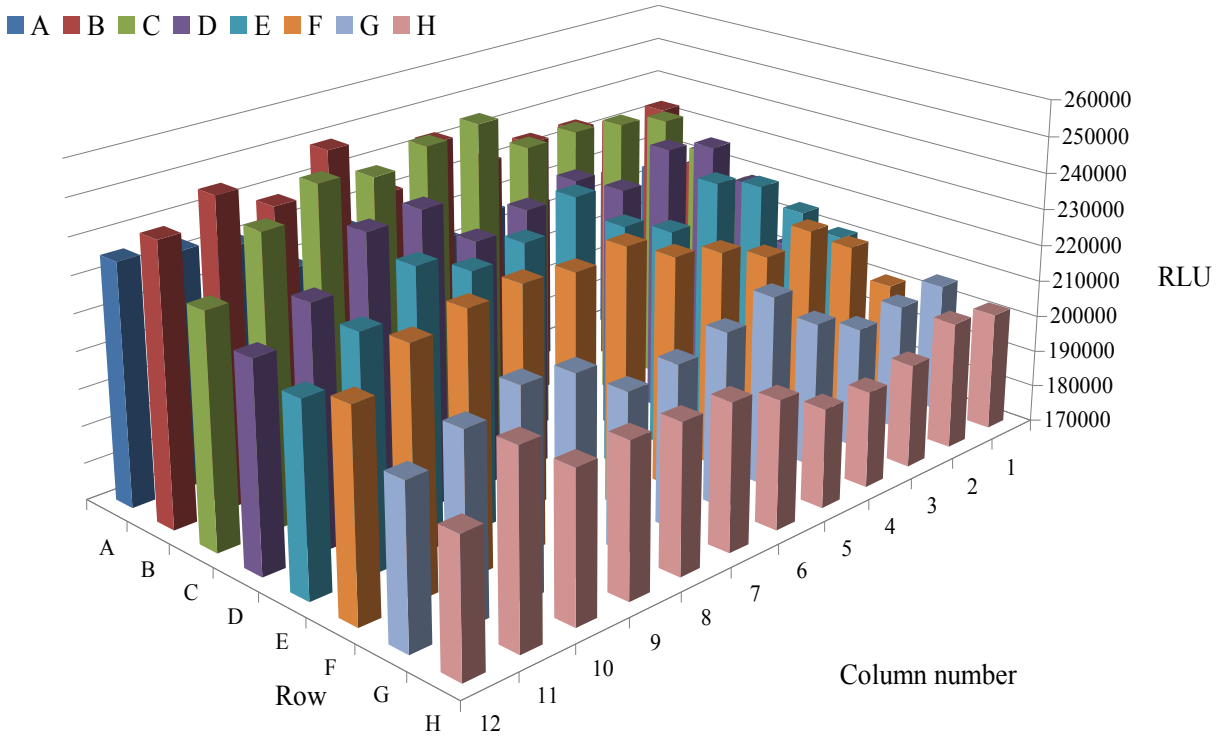

Figure 1: 3D plot of the normalized RLUs, averaged over 6 plates

Statistical calculations were also performed on the rows (A-H), columns (1-12) and 4 circles (indicated in figure 2 by color lines) to asses significant differences between wells on the same plate.

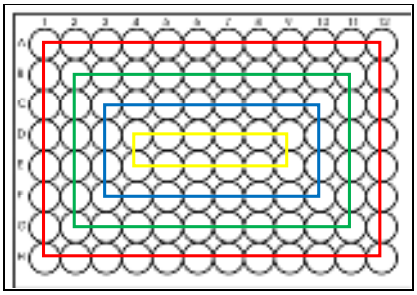

Figure 2: overview of the 4 circles used for statistical calculation

Highly significant differences ($p < 0.001$) were found between the columns and between the rows in a two-way ANOVA. The Holm-Sidak post hoc test showed significant differences (all p-values < 0.001) between the mean RLUs of column 1 and columns 3 to 12 and between column 2 and columns 7, 8, 9, 10 and 11. Significant differences ($p < 0.001$) were also found between row H and all other rows; between row G and rows B, C, D, E and F; between row A and rows B, C, D, E, G; between row F and rows B, C and D; between row C and rows E and D; and between row B and rows D and E.

Significantly different values were also found between the 4 circles (p = 1.43E-12) in a one-way ANOVA. The Ducan's multiple range post hoc test showed significant lower RLU values for the outer circle (red, mean RLU = 215466) compared to the other circles and also for the second circle (green, mean RLU = 229937) compared to the two inner circles (blue and yellow, mean RLUs respectively 242017 and 239907). The CV between the 4 circles was 3.44%, while the CV within was 5.21%.
Since it was obvious from this results that the outer wells on the plate (i.e. rows A and H and columns 1 and 12, or the outer circle) were significantly lower in RLUs than the other wells, the statistical analysis was also carried out for the inner 60 wells of the plate. When considering only the 3 inner circles, the p value was still highly significant (p = 0.002) and the post hoc also showed significantly lower values for the second, green circle compared to the 2 other circles. The CVs, both between and within the circles, were lower with respective values of 2.89% and 5.08%. Concerning the rows and the columns, the same trend was seen: p-values were still highly significant ($p < 0.001$ for both the rows and the columns). In the Holm-Sidak post hoc test 11 two to two comparisons were significantly different for both the columns (only columns 2 and 3 were significant) and the rows (mostly rows G and F were significant).

As can be seen from these results, variability on the CALUX cell plate is a concern. Especially the outer wells give a significant lower response. The authors therefore suggest not the use the outer circle of the 96-well plate to analyze samples. However, it is important to seed these wells with the cell culture and to add medium to the wells before incubation to optimize the growing conditions of the neighboring wells.
Since also columns 2 and 3, and several other rows showed significant different values in a post hoc test, it is recommended to work in duplicate or even triplicate (for calibration solutions, quality controls, blanks and samples) and to spike with at least 3 quality control solutions, spread out over all inner wells, to estimate the maximal variation. Analyzing different dilutions of a sample can also provide a better estimate of the between-well variability.

To reduce the variability on a plate further research is needed to optimize to growth conditions of the cells. Currently, test are performed with the more sensitive H1L7.5c1 mouse cell line[8] and the influence of a lower incubation temperature[9] (33°C) on the variability is investigated using the H1L6.1c3 cell line.

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