# THE CALUX BIOASSAY: A QUEST FOR OPTIMIZATION PARAMETERS TOWARDS A RELIABLE AND COST-EFFECTIVE SEMI-QUANTITATIVE SCREENING METHOD IN DIOXIN POTENCY ASSESSMENT

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## Introduction

The driving force in bioassay analysis of POPs, such as dioxins and dioxin-like PCBs, is to produce quality-full data when used as a semi-quantitative screening method, which is especially the case in official food and feed control where strict regulations exist[1]. The way to obtain such data is by being aware of the influence that key factors can have on your result, acting upon them and keeping them constant over time. Quality of the data can then be reflected as the precision obtained in your results.
Another driving force (applicable to all commercial and educational/academic facilities) is cost reduction of the analytical procedure. This can range from a reduction in media use, luciferase assay reagent, the well size of the cell culture plate (e.g. 96-well) and even to automated dispensing and handling of seeding and dosing actions.
Combining both is the way forward for the CALUX bioassay and thus merits some attention. Emphasis in this study is put on various treatments, general optimizations (quenching, integration time, and edge effect containment) that can easily be implemented to effectively decrease cost and increase sensitivity and precision of the bioassay.

## Materials and methods

CALUX analyses were carried out using the new mouse hepatoma cell line (H1L7.5c1) as described elsewhere[2]. Briefly, cells were maintained in alpha minimal essential medium (α-MEM) supplemented with 10% (v/v) foetal bovine serum (FBS) and seeded in 96-well plates (100µL) at 37°C, 85% relative humidity and 5% $CO_2$. After an incubation time of 24-hours and cells reaching a monolayer, sample extract dilutions and TCDD treatment solutions (both 1% DMSO as final concentration) were dosed in triplicate (100µL). Cells were again incubated over a 24-hour period after which lysis and measurement were performed using Luciferase assay substrate and a Glomax 96-well plate reader (both from Promega, The Netherlands). Data analysis was performed in Excel® where statistical analysis and $BEQ/EC_{50}$ quantification[3] involved fitting the 4-parameter Hill equation or the newer Box-Cox and Slope ratio method (Excel Solver add-in enabled).

## Results and discussion

Initial experiments conducted were pillared on 3 major topics: a) quenching of the luminescent signal for reducing a possible cross-talk effect between neighboring wells, b) assessing the integration time needed for optimal and stable luciferase activity reflected as RLUs (Relative Light Units), and c) managing the edge effect of the bioassay.

### *Quenching*

Quenching refers to the process of decreasing (or in the best case complete removal of) the luminescent signal thus considerably reducing well-to-well crosstalk. Light emitted from a single well on a 96-well plate (luciferase-luciferin reaction) can influence the measurement of that same reaction in a neighboring well through 2 types of light transfer or scatter; reflection and refraction. From experience, we had noticed this phenomenon was most pronounced when a blank sample (DMSO, media …) was placed alongside a high TCDD treatment solution. This problem can either be solved by changing the experimental plate design or by reducing the light present in a single well after measurement. The latter would allow luciferase reagent injection (5.6s lag time), followed by a first measurement (3s integration) of the reporter gene output as RLUs, a second injection with quench reagent (1s lag) and also a second integration (2s) to detect and quantify the remaining light signal. These actions are consecutively executed before moving to the next well. Quenching reagents tested were NaOH

(0.50M standard concentration; personal communication by Dr. J. Haedrich, EURL Freiburg), EDTA (ethylene-diaminetetraacetic acid, saturated solution at ~0.2M) and SDS (sodium dodecyl sulfate, 0.58M standard conc.).

Results are summarized in figure 1. The far left pane is the control treatment where no quenching agent was added. The graph shows that the catalytic conversion of luciferin to oxyluciferin is maintained for at least 3 seconds (1s delay and 2s integration time) after normal measurement and that it loses none of its signal expressed in %RLU. The EDTA quench reagent (middle pane) acts as the most active inhibitor on light production with an average reduction of 99.5% (min. 99 to max. 100%) of the original light signal. The EDTA works as a potent chelator for the $Mg^{2+}$ ions that are used as a co-substrate in the conversion of luciferin to oxyluciferin by the produced luciferase.

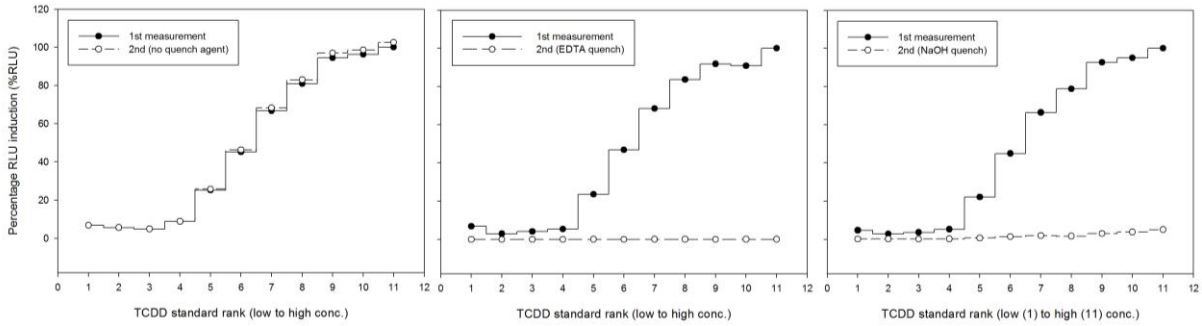


**Figure 1: Effectiveness of various quenching agents (EDTA, NaOH) vs. control treatment.**

The NaOH (far right pane), on average, quenched 98% of the light signal (min 93% to max. 100%). The SDS (data not shown) was less effective with an average 90% quenched (83-97%). Additional problems also arose with the SDS due to foaming (surfactant action) and potential problems with the injector by residue build-up, which was also the case with EDTA. The operating mechanism of NaOH and SDS on the luciferase reaction or constituents present in the mixture remains unknown. It is possible that the NaOH increases pH, thereby affecting the tertiary structure of the protein and causing emission at a different wavelength thus creating a spectral shift. SDS acts as a protein denaturant and possibly affects the protein's tertiary structure and its catalytic capabilities.

*Integration time*

Integration time is the time during which the luminometer integrates the produced light and provides the analyst with RLUs. Photons are detected by a PMT (photo multiplier tube) and are created through the emission of energy when the chemically excited oxyluciferin returns to its ground state[4]. Typically, a delay or lag time is also in effect to reduce flash-type or burst kinetics (where a sudden flash of light is produced and could bias the measurement) and integrate the light produced by a stable reaction. The importance of the integration time is two-fold: (1) given the current delay time (5.6s) are we indeed integrating a stable signal and (2) what is a necessary time of integration? Especially the latter can provide a much faster assay (e.g. 15s compared to 3s over 100 wells equals a time gain of 20min per plate) and will increase daily assay throughput.

Preliminary results indicated saturation of the PMT occurred at non-background response of the cells in kinetics mode. Due to a high refresh rate during data acquisition the luminometer's dynamic range is shortened, resulting in a lowered RLU threshold level during a given time interval (a phenomenon known to the manufacturer). The only measurable data points were the 3 lowest standards with a concentration of $7.6x10^{-4}$ down to $3.0x10^{-6}$ nM (treatment solution), and a DMSO and media blank. Lowering the rate of data acquisition from 10/s to 1/10s did not resolve this problem. Data obtained for the lower TCDD standards are represented in figure 2; kinetic profiles at lower frequencies provide identical information (data not shown). All show the initial flash of light, followed by a small drop and increase in light production. As a rough estimate, signals start to drop after 40 seconds. Care has to be taken regarding the profile in figure 2 (especially the figure on the right). Even though a sharp increase and drop are noticeable, they only occur over a range of 200,000 RLUs between 3.30 and $3.50x10^{6}$ RLUs. Based on the graphs we can visually determine the optimum for both delay and integration time

as being 15s and 10s when searching for the most stable signal on the y-axis. Theoretically, ten seconds (maximum possible integration time on the GloMax 96 luminometer from Promega) will provide an integrated signal encompassing the highest possible amount of data points and smooth out overall variance. Determining the lowest deviation for 100 consecutive data points (10s integration; 10/s frequency) states that the best delay is indeed near the 15 second mark. Comparison of both scenarios (15s + 10s vs. the current 5.6s and 3s) yields, at 4 different frequencies, deviations that are consistently lower when averaged over 10s and after a delay of 15s. Comparing either scenarios or treatments, we obtain a p-value of 0.02 (t-test paired one-sided; equal variance), rejecting the null hypothesis at the 0.05 significance level and accepting it at the 0.01 level. Selecting the minimum and maximum values in the 0-25s time window as a worst case scenario and comparing these for a pairwise t-test, the p-value remains the same 0.02 (paired one-sided).

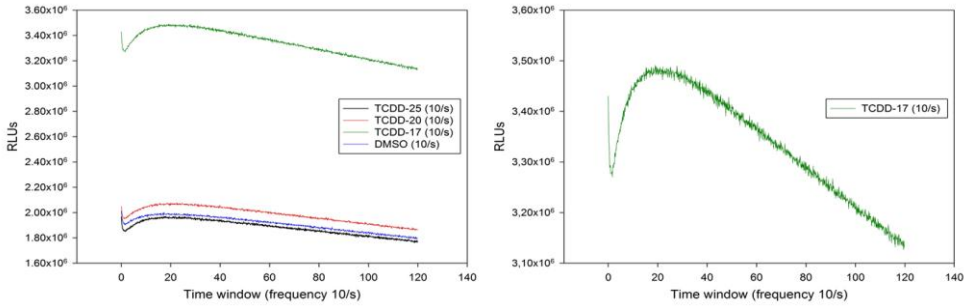


**Figure 2: Kinetic profiles (RLUs) at data acquisition rate of 10/s.**

From the above experiments and resulting data, we can conclude that statistically, a short (5.6 + 3.0s) and a long (15 + 10s) treatment scenario are borderline different from one another and provide similar RLUs. Even when choosing a longer delay time, the integration time can be optimized to be very short  Indeed, for the latter statement, RSDs only rise to 0.24% when integrating the data for 1 second after a 15s delay time. Alternatively, a troublesome issue that remains is the spread on the minimum and maximum RLU values that could occur for more concentrated TCDD standards using the H1L7.5c1 cell line.
In addition, it must be kept in mind that all data originates from one well and that three wells (triplicate dose) are used to constitute a single RLU linked to one concentration level. Also, RSDs of 5% are frequently encountered, thus exceeding the 0.5% precision (maximum value) obtained when assessing the variance in kinetic mode. The reasons for these larger RSDs most likely lie in the analytical equipment for dispensing the luciferase reagent (50µL with a CV of ~1.5%), instrumental error when pipetting lysis, etc. … and in the cell response from well to well thus affecting Michaelis-Menten kinetics and the resulting RLUs.

*Managing the edge effect*

The "edge effect" is an observation in microplates (24, 96, 384, 1536-well) referring to measurements from the outside wells that are often statistically different from wells in the center or near the center of the plate. These values could be higher or lower with respect to the inner wells and depend on the cell type used in such experiments. Hence that some labs do not use any of the outer wells, leaving them blank or filling them with a liquid (water, media …). This "edge effect" has been scarcely discussed in in literature[5, 6], but remains a problem to many scientists. General observations regarding the causes are summarized as (1) the presence of a thermal gradient, (2) evaporation rate of liquids, (3) well density (96 to 1536) and (4) the (in)homogenous nature of cells in a single well.

Based on previous results and on literature, we initialized the use of a thin copper plate (approx. 40x40cm) on top of the existing incubator shelf. This implementation is two-fold: (1) it reduces potential bacterial contamination and (2) its' heat conducting properties are expected to result in an even growth of cells and most importantly, and even temperature throughout the plate bottom. Furthermore, seeding cells and pre-incubating them outside the incubator have been shown to reduce thermal gradients in peripheral wells between dispensing plates and cell adhesion to the bottom surface[6]. Additionally, the implementation of BREATHseal™ (henceforth referred to a as simply BREATHseal) technology (Cat No. 676051; Greiner Bio-One) was also investigated. This BREATHseal is a gas-permeable and piercable membrane consisting of heat-sealed rayon fibers. Pore sizes

vary between 10-50µm and are layered in such a way that applying the membrane acts as a filter for any airborne bacteria while still remaining optimal oxygen supply. Applying the membrane or seal would result in an even evaporation of water from the media and could cancel out any well-to-well variation by a change in osmolality

Verifying the control setup and comparing this with the BREATHseal treatment indicates an overall reduction of the RSDs associated with a given triplicate. Media and DMSO blanks struggle to reach the 10% RSD as do the lower TCDD standards (equaling to roughly the same induction percentage as DMSO). Repeating the experiment, however, we obtain all RSDs equal to or below the 5% RSD mark (exempt DMSO and media blanks). This is verified with the H1L7.5c1 cell lines (figure 3). With regards to EC50 values, no differences are observed when applying the BREATHseal for the H1L7.5c1 cell line (two-sided t-test p-value of 0.67). Values obtained were 0.186 pg TCDD (control; n:6) vs. 0.181 pg TCDD (BREATHSeal treatment, n:6).

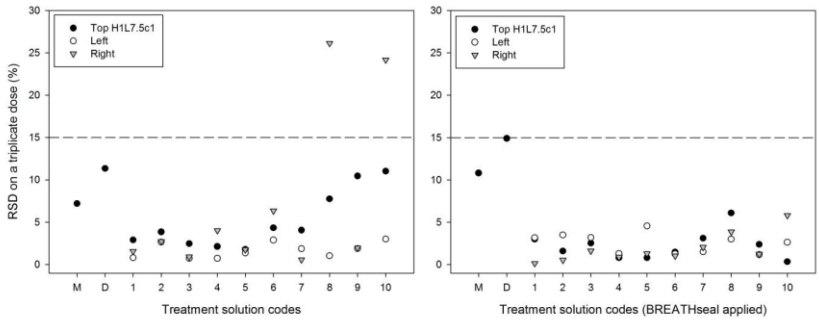


**Figure 3: Variance stabilization using BREATHSeal (right) over regular condensation lids (left),TCDD is dosed in the top (row A-C), left (D1-H6) and right (D7-H12) section of a 96-well plate.**

Executing a single factor ANOVA (analysis of variance) in which the 3x3 treatments at a single dose level on the same plate (and in essence the location or well dosed) are compared to one another yields a p-value of 0.999 for the 7.5c1 cell line. These data ultimately suggest that the whole plate can be used, without any positional effect on cell response. Furthermore, since TCDD curves behave very similar in the given layout of dosing, sample dilutions should provide true and unbiased values relative to a TCDD standard.

Based on all executed experiments, we were able to introduce a preventive measure for well-to-well crosstalk by adding a quenching agent (NaOH). Additionally, lag time (5.6s) and integration time (3s) were and can be kept as short as possible to increase assay throughput (saving valuable time). At the precision level of the assay, we were able to keep variances of triplicate analysis down and consistent (by means of BREATHSeal application) throughout the whole 96-well plate and at various dose concentration levels, thereby effectively justifying the inclusion of the outer edge and reducing costs on a sample throughput basis. It's envisioned that all of these findings, especially those keeping down variances, increase reliability of the assay, decrease cost, and could pave way to analysis using higher density well plates at a precision level comparable to that of current 96-well plates.

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**References:**
1. Haedrich J, Stumpf C, Podestat U, Kotz A, Wahl K, Malisch R. *Organohalogen Compd* (2012); **74**: 177–81
2. Van Langenhove K, Mihale M, Croes K, Tungaraza C, Denison M, Baeyens W *et al. Organohalogen Compd* (2013); **75**: 530–3
3. Elskens M, Baston DS, Stumpf C, Haedrich J, Keupers I, Croes K *et al. Talanta* (2011); **85**: 1966–73
4. Fan F, Wood K V. *Assay Drug Dev Technol* (2007); **5**: 127–36
5. Gordon JD, Denison MS, Chu AC, Matherly C, Clark GC. *Organohalogen Compd* 2007; **69**: 1249–52
6. Lundholt BK, Scudder KM, Pagliaro L. *J Biomol Screen* (2003); **8**: 566–70