INTRODUCTION OF A LINEAR 2,3,7,8-TCDD CURVE-FIT AS A MEANS TO IMPROVE PERFORMANCE CHARACTERISTICS OF THE DR CALUX® BIOASSAY

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Introduction

In DR CALUX® cells, the fire fly luciferase gene is linked to a set of dioxin responsive elements (DREs) which control AhR mediated expression of the luciferase gene. This enables to quantify the dioxin-like toxic potency of a sample with a luminometer. After incubation of DR CALUX® cells with the sample, the amount of light emitted by induced luciferase activity is interpolated in a sigmodial curve-fit of a concentration series of 2,3,7,8-TCDD (Figure 1A).

As stated in the harmonized quality criteria for bioassays analyses of PCDD/Fs in feed and food, the light induction of the sample dilution used for quantitation should be within the linear portion of the response curve and above the limit of quantitation[1,2]. As indicated in figure 1B, this suggested range of quantitation is between 1 and 3 pM 2,3,7,8-TCDD. By using multiple sample dilutions, only results with responses in the defined quantitation range are selected.

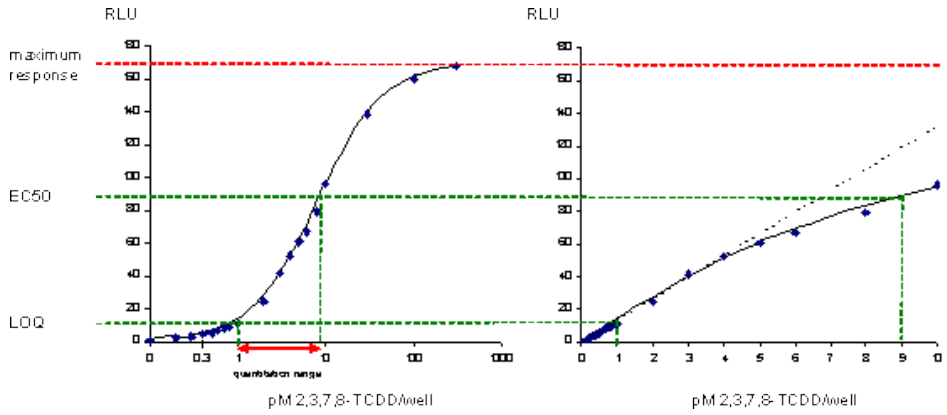

Figure 1 Graphical representation of a typical response of DR CALUX® cells to 2,3,7,8-TCDD (A = linear – log scale; B = linear – linear scale). Saturation of the binding capacity of Ah-receptor by PHAHs results in a maximum response. The EC50 is the concentration which results in 50% of the maximum response. The slope of the sigmodial curve reflects the ratio of PHAH/Ah-receptor binding (Hill coefficient =1). The Limit Of Quantitation (LOQ) is the concentration 2,3,7,8-TCDD with a quantifiable response. For TEQ determination of samples, dilutions of samples are incubated under the same circumstances as the 2,3,7,8-TCDD concentration series.

The standard 2,3,7,8-TCDD calibration range is used during the iteration process for optimization of the curve-fit. Interpolation of a 1 pM 2,3,7,8-TCDD concentration in the fitted curve results in an average concentration of 1.0 pM ± 27% standard deviation (n = 115, randomly selected over a period of 3 years). Since the requirements for the DR CALUX® bioassay to determine dioxin levels at ever decreasing levels in a wide variety of matrices, the reproducibility of the fit in the lower region of the calibration range is of utmost importance. To obtain a higher reproducibility between the LOQ and the 3 pM 2,3,7,8-TCDD concentration in the DR CALUX® bioassay, the use of a linear fit in comparison to a sigmodial fit was evaluated.

Methods and materials

2,3,7,8-TCDD calibration range For the standard 2,3,7,8-TCDD calibration curve used for sigmodial curve-fitting,

the following stock solutions of 2,3,7,8-TCDD in DMSO were prepared: 0, 37.5, 125, 375, 1250, 3750, 1250, 3750 pM. Two new stock concentrations of 2,3,7,8-TCDD in DMSO were added: 75 and 250 pM. The final concentrations of 2,3,7,8-TCDD in exposure medium (0.8% DMSO) are respectively 0, 0.3, 1.0, 3.0, 10, 30, 100, 300 pM/well for the standard calibration range and 0.6 and 2 pM/well for the additional calibration concentrations.

DR CALUXâ bioanalysis The procedure for the DR CALUXâ by BDSbioassay is described in detail previously[3]. Briefly, the bioassay is performed using a rat hepatoma H4IIE cell line stably transfected with an AhR-controlled luciferase reporter gene construct. Cells were cultured in α-MEM culture medium supplemented with 10% ($^{v}/_{v}$) FCS under standard conditions (37^{o}C, 5% CO_2, 100% humidity). Cells were exposed in triplicate on 96-well microtiterplates containing the standard 2,3,7,8-TCDD calibration range, the additional 2,3,7,8-TCDD calibration concentrations, a DMSO blank, an internal reference material and various samples extracts at multiple dilutions (e.g. sediment, foodstuffs, feedingstuffs). Following a 24 hour incubation period, cells were lysed. A luciferine containing solution was added and the luciferase activity was measured using a luminometer equipped with 2 dispensers. Overall, 128 sample extracts were analyzed using 67 microtiter plates.

Table 1 Average, median and relative standard deviation of 0.3, 0.6, 1.0, 2.0, 3.0 pM 2,3,7,8-TCDD standards and IRM following interpolation of analysis results in the sigmodial fit and linear fit

[2,3,7,8-TCDD]	**Sigmodial curve fit**			**Linear curve fit**		
(pM/well)	AVG	median	%SD	AVG	median	%SD
0.3	0.25	0.24	37	0.33	0.32	25
0.6	0.50	0.53	36	0.59	0.59	15
1	0.92	0.90	15	1.0	1.0	10
2	1.8	1.8	20	1.9	1.9	13
3	3.0	3.0	6.8	3.1	3.1	4.6
IRM	2.0	2.0	15	2.1	2.1	12

*Evaluation of analysis results*Sample analysis results were interpolated in both a sigmodial fit and a linear fit. The sigmodial fit was constructed using the DR CALUX® responses of the 2,3,7,8-TCDD calibration series (0.3, 1.0, 3.0, 10, 30, 100, 300 pM 2,3,7,8-TCDD/well). The linear fit was constructed using the DR CALUX® responses of 0.3, 0.6, 1.0, 2.0 and 3.0 pM 2,3,7,8-TCDD standards/well.

Results and Discussion

The validation of the DR CALUX® bioassay has been described in detail elsewhere[3,4,5].In the present study, the percentage standard deviation of the 1 pM 2,3,7,8-TCDD concentration was 15% following interpolation in the sigmodial fit. In contrast, the percentage standard deviation of the same 2,3,7,8-TCDD concentration in the linear fit was found to be 10% (Table 1). Furthermore, the percentage standard deviation of the 3 pM 2,3,7,8-TCDD standard and the IRM, both used for quality control purposes, decreases when interpolating analysis results in the linear fit as compared to the sigmodial fit. This shows that using a linear fit improves the reproducibility of analysis results. Improvement in reproducibility is especially profound between the LOD (0.3 pM/well) and LOQ (1.0 pM/well) of the DR CALUX® bioassay.

In figure 2, the correlation between the DR CALUX® sample analysis results following interpolation in the sigmodial fit and interpolation in the linear fit, is given. A good correlation (R^2 = 0.99) between either methods for quantitation of analysis results, in addition to a linear regression coefficient of 1.03, shows that both methods give equal results.

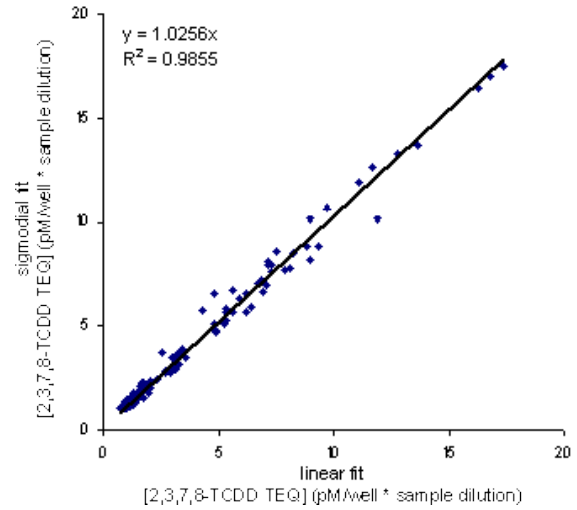

Figure 2 Comparison between sample analysis results following interpolation in sigmodial fitted and linear fitted 2,3,7,8-TCDD calibration series.

Based on the present findings, we suggest an adapted 2,3,7,8-TCDD calibration range and to use a linear fit prior to interpolation of samples analysis results. In figure 3, a suggested microtiterplate setup is given. Furthermore, the use of a linear fit gives reason to re-evaluate the LOQ and hence lower the present LOQ.

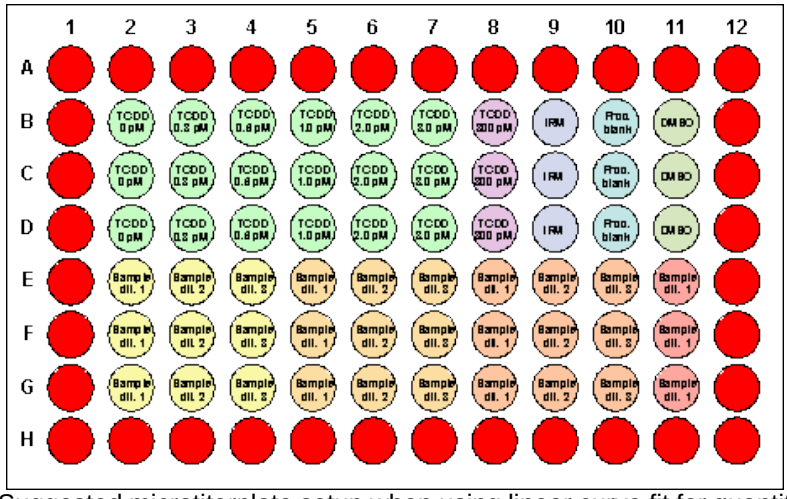

Figure 3 Suggested microtiterplate setup when using linear curve fit for quantitation of analysis results

References

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