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The CALUX bio-assay: Analytical comparison between mouse hepatoma cell lines with a low (H1L6.1c3) and high (H1L7.5c1) number of dioxin response elements

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ABSTRACT

Dioxins, furans and polychlorinated biphenyls are contaminants of high concern and as such, sensitive tools are needed to detect these persistent organic compounds in a variety of matrices. Due to the large amount of samples that need to be investigated for example for food and feed control, the CALUX bioassay (H1L6.1 clone) was developed allowing rapid and cost-efficient analysis of biological and environmental samples. Recently, a new and more sensitive clone (H1L7.5) was constructed as the third generation CALUX bioassay. This new cell line was subject of an amplification of dioxin response elements (DREs), allowing lower concentrations of target compound to be analyzed. A comparison is made between the previous, well-defined H1L6.1c3 cell line and the new H1L7.5c1 cell line: it appears that the bioassay making use of the higher number of DREs is more stable and robust, shows better repeatability and reproducibility and is; on average, 3 times more sensitive.

1. Introduction

Polychlorinated dibenzo-*p*-dioxins, -furans and biphenyls form a group of ubiquitous environmental contaminants that persist in time as well as accumulate in a non-polar matrix and further up the trophic chain [1]. These compounds have been found in environmental samples worldwide, in food and feed and in human body fluids. The non-polar character of the dioxin like compounds leads to their accumulation in the lipophilic parts of the sample matrices. Due to the potency of the most toxic congeners, the tetra and penta chlorinated dibenzodioxins and furans, these compounds can already exert health effects at very small amounts. It is therefore important to have sensitive tools that allow quantification of these low amounts of toxic compounds in biological and environmental samples.

Most commonly and traditionally, GC–HRMS (Gas Chromatography–High Resolution Mass Spectrometry) has been the golden standard and reference method [2]. However, this reference method only focuses on 29 congeners, of in total 419, taken up in the 2005 WHO-TEF concept [3], because they were the most toxic. All dioxin like compounds are persistent and bio-accumulative and have a planar aromatic structure and the ability to bind to the Ah (aryl hydrocarbon) Receptor. The Ah receptor is a high affinity, low capacity binding multimeric protein complex that exhibits saturable binding with TCDD and other aromatic and halogenated aromatic hydrocarbons [4], where it acts as a transcription factor. Due to the molecular dimensions of the Ah receptor, i.e. a rectangle of approximately 3 Å × 10 Å with a maximum dimension of 14 Å × 12 Å × 5 Å [5], the most active congeners are those that display substitution in the lateral positions such as the prototypical 2,3,7,8-TCDD (2,3,7,8-tetrachloro-dibenzo-*p*-dioxin).

The aryl hydrocarbon receptor is the key in mediating the toxicity of dioxins and dioxin-like compounds [6]. The pathway is briefly described in the supplementary material of this paper. Measuring the concentrations of target compounds in samples, multiplying them with the corresponding TEF values and making the summation of all individual toxicities for the 29 WHO-compounds, provides a WHO-TEQ (World Health Organization Toxic Equivalence).

In contrast to the purely chemical measurement method, a biological assay exists for the screening of a variety of matrices for their biological potency equivalence. The CALUX (Chemically Activated Luciferase gene expression) bioassay uses a recombinant mouse hepatoma cell line [7], starting from the Hepa1c1c7 wild-type mouse cell line, that has been stably transfected with a luciferase reporter gene. The resulting light response is given in

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an AhR-, time-, dose- and ligand-specific manner [7,8]. All compounds that exhibit similar structural properties to that of TCDD and are persistent in time can elicit a response with this bioassay.

As such, the bioassay gives no specific information about congeners or homologues but integrates the responses of each of the AhR ligands present in the sample and provides an overall Toxic Equivalent value. Comparing the WHO-TEQ value resulting from the GC–HRMS measurement with the CALUX result reveals that there exist some discrepancies between both methods [9,10]. These discrepancies are generally, however, not exhaustive; CALUX provides the higher value of both methods because the CALUX response includes all ligands binding to the AhR while the GC–HRMS result is limited to the 29 WHO-compounds.

When the sample matrix becomes increasingly complex, it often happens that non-additive interactions [9,11] occur with the CALUX bioassay. Practically, the effect of non-additive interactions is strongly reduced by performing a clean-up step and a separation of target compounds in a PCDD/F fraction and a PCB fraction to remove essentially the PAH compounds [12] and to eliminate the antagonistic effects between PCBs and PCDD/Fs. This means that the additivity principle for the target compounds is now valid and allows a better agreement between the GC–HRMS and CALUX bioassay results. On the other hand, the CALUX bioassay provides a rather biological response with respect to the chemicals present in the sample [11]: this is an important feature because in case the GC–HRMS and CALUX bioassay provide a different response, the scientist has to decide which of both results would be included in a risk assessment. To highlight that there are differences between GCMS-TEQ and the CALUX responses (although they are basically very complementary), the term CALUX-BEQ (Biological Equivalence) has been adopted [13,14] to stress better the biological nature of the CALUX bioassay response and its implications for human health.

The CALUX bioassay has been proven to be a valuable tool for screening dioxin and dioxin-like compounds in biological and environmental samples and this at a high rate and low cost. However, in many samples the dioxin and dioxin-like PCB levels are very low, sometimes below the detection limit of the H1L6.1 cell line. In other cases the amount of sample is so limited (for example blood samples) that it does not allow to separate and quantify the PCDD/F and PCB fractions and/or to carry out sufficient repeats to determine the uncertainty on the result. Recently a new, more sensitive cell line, H1L7.5, was developed but no evaluation of the analytical characteristics of this cell line has been made.

The objectives of this paper are to establish a dataset for both (H1L6.1c3 and H1L7.5c1) mouse hepatoma cell lines (accuracy, repeatability and detection limit) in which the second generation 6.1 cell line has already been extensively researched [7,9–11,15–18], and extending this into the newer, third generation 7.5 cell clone for reasons of comparison. Standard solutions as well as a number of natural sediment samples were analyzed with both cell lines.

2. Materials and methods

2.1. Cell line evolution

The CALUX bioassay utilizes a reporter gene that is typically joined to a promoter sequence in an expression vector and then transferred into cells. Ideally, such reporter genes are not endogenously expressed in the cell and are applicable to assays that are sensitive, quantitative, rapid, easy, reproducible and safe. Here, luciferase was chosen due to the advantage luminescence has over fluorescence, accomplished by lower background noise.

The most common luciferase genes are those obtained from the click beetle and the firefly, both belong to the same luciferase family, and from *Renilla reniformis* (sea pansy). The most cell lines described in literature make use of the firefly luciferase [19].

The gene encoding the firefly luciferase is called *luc*, and is a monomer that requires no post-translational modifications, meaning that it is a mature enzyme directly upon translation from its mRNA. The earliest vector used was the pGudLuc1.1 (US patent 5,854,010) that contained the firefly luciferase under control of the MMTV LTR (Mouse Mammary Tumor Virus-Long Terminal Repeat) viral promoter and a fragment from the 5′ upstream region of the CYP1A1 gene.

2.1.1. H1L6.1c3 cell line

Over the years, improvements were made to the constructs and new, stable cell lines were created such as the H1L6.1c3 clone that made use of the same wild type Hepa1c1c7 cell line, but a different vector. Instead of the pGudLuc1.1, the pGudLuc6.1 vector was inserted. This vector originated from the pGL3-Basic plasmid (as opposed to the pGL2) and has a more stable luciferase protein that, in addition, was targeted to the cytosol and not to the peroxisomes after translation.

The largest difference is seen on the level of the dose–response curves. The stably transfected H1L6.1c3 cell line is approximately seven times more sensitive than the first generation H1L1.1c2 [15].

2.1.2. H1L7.5c1 cell line

Recent developments included a significant upscaling in the amount of DREs used in the luciferase vector and hence an increase in the sensitivity and responsiveness of the assay [13,20].

The pGL7.0 vector, with multiple dioxin responsive domains (DRDs), was inserted, with each domain containing 4 DREs from the pGudLuc6.1 vector. Resulting plasmids were named pGL7.*X* with *X* representing the numbers of DRDs (a cell line with 5 DRDs provided the best characteristics). The current second generation plasmids, such as the pGudLuc6.1, have magnitude of induction responses somewhere in the vicinity of 7000 relative light units (RLUs) per mg protein according to literature. These third generation variants range from 10,000 to 60,000 RLU per mg protein [20].

The larger luciferase activity at lower concentrations of TCDD results in a lower detection limit and allows more accurate determinations in this low end of the concentration curve [21]. This 'new' CALUX bioassay was primarily created for analysis of matrices with low concentrations of dioxin and dioxin-like compounds such as blood and serum samples.

2.2. Reagents and chemicals

Dimethylsulfoxide (DMSO) was purchased from Merck (Pro analysi ACS, Germany) and used to prepare stock and standard solutions of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (2,3,7,8-TCDD, 99% purity) as supplied by Campro Scientific (Cambridge Isotope Laboratories CIL-ED-901).

Biological formulae were purchased from Invitrogen (Life Science Technologies, UK), including α-MEM, FBS (South American Origin) as well as trypsin (0.25%; stock solution) and 10× PBS (Ambion).

2.3. Analysis methods

Both mouse hepatoma cells were grown to near confluence (roughly 85–90% coverage of the tissue culture plate) in the following media solution (henceforth referred to as media): 90% α-MEM (Gibco® Invitrogen, UK) supplemented with 10% FBS (South American; Gibco, UK). Cells were harvested by adding a 0.05% trypsin-EDTA solution and gentle swirling of the plate to detach

cells. Cells were transferred to conical tubes with fresh media and centrifuged at 1100 rpm. The supernatant was removed and cells were resuspended in a known volume of media. A haemocytometer (Neubauer) was used to count the cells present in the media solution.

Cells were seeded into clear bottom 96-well plates (Perkin Elmer, USA) by adding 100 μl of a homogenous cell suspension at a cell density of 7.5×10^5 cells/ml using a 12-channel multipipettor (Brand). Subsequently, cells were incubated for 24 h at 37 °C in 5% CO_2 and 80% relative humidity. After removal of the media solution, cells were dosed with diluted TCDD standard solutions. Table 1 presents the initial TCDD standard solutions in DMSO prior to dilution; they range from 10 pM to 1 μM. This initial TCDD standard solution in DMSO is 100-fold diluted with media solution (4 μl TCDD in DMSO and 396 μl media) and 100 μl of this diluted solution was added to the wells in triplicate. The final percentage DMSO was 1% (v/v). A DMSO treatment blank is made in the same way (4 μl of pure DMSO in 396 μl media solution) and added as a negative control. A media blank is also added (contains no DMSO and consists solely of media). Final sample extracts in DMSO are treated in the same manner as the TCDD standard solutions: target analytes present in 4 μl of sample extract in DMSO are mixed with 396 μl media and 100 μl of this mixture is analyzed in triplicate. In this paper we will discuss the results obtained on sediment samples. The complete treatment procedure of the samples (extraction, clean-up and separation of the PCDD/F and PCB fractions) is described elsewhere [14].

Finally, cells were incubated for another 24 h, the wells were rinsed with 50 μl of 1× PBS (Gibco, UK), checked for viability and any altered morphology under the microscope, followed by addition of 50 μl of 1× lysis buffer (Promega, USA).

Afterwards, wells were read using a Glomax 96-well plate luminometer (Promega, USA) with a 5.6 s incubation (lag) time for both cell lines, after injection of 50 μl of the reconstituted luciferase assay buffer. For both cell lines, values are reported as the percentage of maximum RLU induced by 2,3,7,8-TCDD.

2.4. Statistical methods

To be able to use the CALUX bio-assay as a semi-quantitative measuring technique [22,23], a calibration curve with the Hill equation (see below) fitted through the data points using an weighted least squares regression is implemented as described [18]. Standard curves produced by bio-assays like CALUX resemble a sigmoid form (light response against the concentration of TCDD dosed) which makes the fitting process more complicated. The 4-parameter Hill equation [18,24] is most often used:

$$\text{RLU} = \frac{v \cdot d^n}{d^n + k^n} + b + \varepsilon \qquad \text{(Hill equation)}$$

where d is the TCDD concentration or mass unit, v is the limiting value of the RLU response as the concentration of TCDD increases, k is the dose corresponding to 50% of the maximum dose response, n is a parameter that defines the sigmoid shape of the curve and b is the intercept parameter. The residual term is represented by ε.

Once the sigmoid Hill equation has been derived from the standard solutions, inverse prediction allows quantifying the dioxin concentration in an unknown sample in a pg CALUX-BEQ/g sample manner. Either an inverse prediction can be performed on single point estimates in case of low contaminated samples, or full dose-curves (0–100% induction) can be produced for more contaminated sediment samples.

An analytical comparison between the 2 cell lines will be carried out including their EC_{50}-values (effective concentration), repeatability and reproducibility. The individual plate responses at 20, 50 and 80% induction were arranged day by day and the repeatability (within day variability) and reproducibility were calculated using a one-factor ANOVA protocol.

The formula used to determine the within-lab reproducibility (S_R^2) was $S_R^2 = S_L^2 + S_r^2$. The S_r^2 denotes a pooled repeatability variance and the S_L^2 provides a measure of the between day variance [25,26].

Additionally, a functional performance criterion using a power relationship [18] between the standard deviations (stdev) and the response signal (in %RLU) can be obtained and provides the reproducibility standard deviation s_R for an entire dataset by the following general equation: $s_R = 10^{\text{Intercept}} \times (\%\text{RLU})^{\text{Slope}}$.

Emphasis will be put on reporting the relative induction levels and their treatment concentrations expressed in picomolar units (i.e. pM TCDD). In addition, it is possible to bridge literature data that has been historically presented in molar units [15,16] to the present, more commonly used, mass-base unit.

3. Results and discussion

3.1. Dataset

In the period June to August 2010, a series of analytical experiments were carried out. In total, 16 and 12 plates were analyzed with, respectively, the 6.1 and 7.5 cell lines. The plates were selected for their identical plate lay-out, concentrations and general treatment conditions that remained constant over the course of time. During preliminary data analysis, triplicate measurements that cause the intra coefficient of variation (intra-CV, %) to exceed 20% are re-evaluated. The deviating result is then deleted and the CV related to the 2 remaining measurements is checked: if it is <20% the 2 measurements are included in the dataset, Table 1 provides more general information on the dataset and the total number of measurements included within the data analysis. If all triplicate measurements would have satisfied the <20% intra CV criterion, the maximum number of measurements (Table 1) for the 6.1 cell line should be $N = 48$ and for the 7.5 cell line $N = 36$.

It can be noticed here that, for the 6.1 cell line several measurements had to be rejected, especially for the lower standards (up to 5.04×10^{-11}) using the <20% CV criterion, while it is much less pronounced for the 7.5 cell line. This is most likely due to the fact that with the 6.1 cell line standard 1 is situated in the lower plateau of the sigmoid standard curve (see further in this paper) and as such (when using the CV concept) combines a high absolute standard deviation (stdev) with a low measurement value. Moreover, it is known that the data [18] are heteroscedastic and therefore the intra-CV is higher in the lower range of the curve. Since the 7.5 cell line provides a much higher signal (more luciferase is transcribed and translated) the CV value at this level of the calibration curve is much smaller.

3.2. Fold induction and RLU comparison

The difference between the two cell lines has very much to do with the ability of the newer cell line (7.5) to produce a remarkably higher output of light (i.e. the higher transcription of the reporter gene that ultimately provides the luciferase protein production due to the strongly increased number of DREs on a DNA strain). This leads to stabilization of the response signal and a lower variance for that result at a given standard concentration of TCDD.

Table 2 shows both of the cell lines with the minimum and maximum response values for the standards used, the fold induction (definition is provided further on in this section) as well as values for DMSO and media blanks.

All values presented in relative light units (RLUs) are raw data provided by the luminometer. RLU values show higher variation for

Table 1
Number of total measurements (*N*) ultimately taken up in the dataset used for the data-analysis (maximum *N* = 48 (16 triplicates) for the 6.1 cell line and *N* = 36 (12 triplicates) for the 7.5 cell line).

Standards	Location on plate	6.1 cell line		7.5 cell line	
		N	Standard TCDD concentration (M)	*N*	Standard TCDD concentration (M)
1	1 A-C	33	1.01×10^{-11}	36	1.01×10^{-11}
2	2 A-C	40	5.04×10^{-11}	33	5.04×10^{-11}
3	3 A-C	42	3.53×10^{-10}	35	1.51×10^{-10}
4	4 A-C	43	8.57×10^{-10}	35	3.53×10^{-10}
5	5 A-C	46	1.82×10^{-09}	36	5.05×10^{-10}
6	6 A-C	47	3.03×10^{-09}	36	8.57×10^{-10}
7	7 A-C	47	8.08×10^{-09}	35	1.82×10^{-09}
8	8 A-C	47	2.53×10^{-08}	35	8.08×10^{-09}
9	9 A-C	47	1.01×10^{-07}	35	2.53×10^{-08}
10	10 A-C	47	1.01×10^{-06}	35	1.01×10^{-07}
DMSO blank	11 A-C	42	Contains no TCDD	36	Contains no TCDD
Media blank	12 A-C	43	Contains no TCDD	35	Contains no TCDD

the 6.1 cell line (inter-CV between 29 and 40%) than for the 7.5 cell line (inter-CV ranging between 25 and 12%). Contributing factors to an increase in the inter-CV (when comparing plates over multiple days with the raw RLU format) is the dependence of these raw RLU values on parameters such as duration of the experiment, incubation temperature, initial cell count and instrumental limitations (variability and scale limits), despite the fact that these parameters are kept identical as much as possible by the operator. Raw RLU data as such (in its current form of absolute values retrieved from the measurement device) will not be used to compare multiple plates due to the too large variation in scale (maximum vs. minimum value).

Each plate therefore is rescaled: the highest TCDD response in RLUs is set equal to 100%. For a better comparison of the 2 cell lines we kept the background induction unchanged (integral part as a parameter in the Hill equation). All other results are then scaled percentage-wise to this highest value allowing inter-plate comparison and background induction.

From Table 2 we can see that the maximum RLU output is much larger for the new 7.5 cell line, with values reaching up to 30 million RLUs as opposed to the 160 thousand RLUs for the established 6.1 cell line. The minimum values (lowest standard) show a similar trend but provide more information when formatted in a percentage TCDD induction manner. In that case, the minimum value for the 6.1 cell line is almost half of the 7.5 cell line (3.44% vs. 6.76% TCDD induction). The higher amount of DREs present in the new cell line causes the background or lower plateau level of the calibration curve (see Section 2.4) to be higher for this cell line. The same is true for the media blank solutions while the DMSO blank solutions show similar values for both cell lines. The fold induction is defined in several ways, but the most used one [20] is the following: the ratio of maximum TCDD induction to that of the DMSO control. The fold induction based on this definition is very similar for both cell lines (Table 2).

It is also interesting to compare the responses from the lowest standard, the DMSO blank and the media blank. A discrepancy can be observed between the lowest standard and DMSO blank (1%, v/v DMSO/media) values since under normal conditions, the lowest standard should yield a higher or at least an equal response to that of the DMSO blank, the latter being void of any TCDD. Further investigation is needed to clarify this observation. Moreover, the lower plateau level induction matches that of media blanks (3.66 ± 0.91 and 3.44 ± 0.80% induction for the 6.1 cell line vs. 6.76 ± 1.00 and 5.09 ± 0.78% induction for the 7.5 cell line). We also noticed that

Table 2
Overview of assay-specific parameters for both cell lines. The two lowest (identical for both cell lines) and three highest (different among the 6.1 and 7.5 cell line) TCDD concentration standards are given. Values are compared over a total of n plates for the corresponding cell line.

		n	6.1 cell line		*n*	7.5 cell line	
			Average ± stdev	Inter-CV (%)		Average ± stdev	Inter-CV (%)
Standard 1.0×10^{-11} M	RLU	16	5300 ± 1500	(28.7%)	12	2,125,000 ± 530,000	(24.9%)
	TCDD Induction (%)	16	3.5 ± 0.8	(23.4%)	12	6.8 ± 1.28	(18.8%)
Standard 5.0×10^{-11} M	RLU	16	5700 ± 1800	(31.8%)	11	2,900,000 ± 1,300,000	(44,5%)
	TCDD Induction (%)	16	3.7 ± 0.8	(21.8%)	11	8.2 ± 0.6	(7.6%)
8.0×10^{-9} M (7.5)	RLU				12	30,970,000 ± 3,570,000	(11.5%)
	TCDD Induction (%)				12	99.4 ± 0.9	(0.9%)
2.5×10^{-8} M (6.1)	RLU	16	157,000 ± 58,000	(37.0%)			
	TCDD Induction (%)	16	97.8 ± 3.3	(3.4%)			
2.5×10^{-8} M (7.5)	RLU				12	30,120,000 ± 3,800,000	(12.6%)
	TCDD Induction (%)				12	96.6 ± 3.4	(3.6%)
1.0×10^{-7} M (6.1)	RLU	16	155,000 ± 60,000	(38.7%)			
	TCDD Induction (%)	16	96.4 ± 2.2	(2.2%)			
1.0×10^{-7} M (7.5)	RLU				12	30,440,000 ± 3,910,000	(12.8%)
	TCDD Induction (%)				12	97.7 ± 2.7	(2.7%)
1.0×10^{-6} M (6.1)	RLU	16	160,000 ± 64,000	(40.1%)			
	TCDD Induction (%)	16	99.0 ± 1.6	(1.6%)			
Fold induction	Minimum	16	10	-	12	9	-
	Maximum	16	16	-	12	14	-
	Average	16	13 ± 2	(13.2%)	12	11 ± 2	(15.26%)
Media	RLU	16	5600 ± 3400	(61.1%)	12	1,720,000 ± 670,000	(39.0%)
	TCDD Induction (%)	16	3.7 ± 0.9	(24.8%)	12	5.1 ± 0.8	(15.3%)
DMSO	RLU	16	11,000 ± 5000	(44.5%)	12	3,030,000 ± 630,000	(20.9%)
	TCDD Induction (%)	16	7.6 ± 1.0	(13.8%)	12	9.7 ± 1.3	(13.4%)

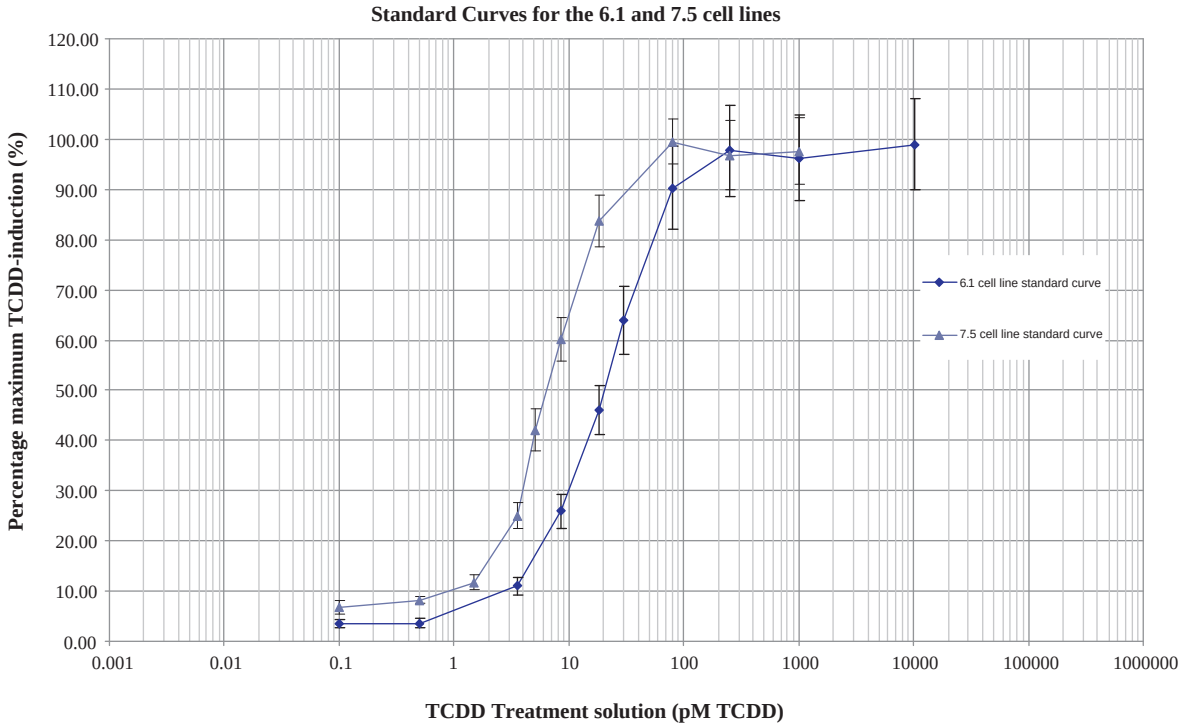

Fig. 1. Standard calibration curves for the 6.1 ($n = 16$) and 7.5 ($n = 12$) cell lines. Standard deviations in % induction are marked by the error bars.

Table 3
Effective concentration values (pM TCDD) at 20, 50 and 80% induction. Averaged values.

	n	6.1 cell line		n	7.5 cell line	
		pM TCDD ± Stdev	Inter-CV (%)		pM TCDD ± stdev	Inter-CV (%)
EC (20)	16	7.6 ± 0.6	(7.9%)	12	3.5 ± 0.4	(10.8%)
EC (50)	16	20.4 ± 2.0	(9.8%)	12	6.9 ± 0.4	(5.2%)
EC (80)	16	54.8 ± 7.3	(13.2%)	12	14.0 ± 1.2	(8.5%)

the DMSO blank results were consistent throughout the individual plates and this for both cell lines.

In Fig. 1 the averaged standard curves for both cell lines are plotted. The inverse prediction, starting from the induction of an unknown sample extract (a number of RLUs), and calculating an amount of TCDD (pg/g CALUX-BEQ for samples) is possible once the calibration curve equation is established. This means that we can determine the TCDD concentration (molar) corresponding to a given induction percentage, such as at 20% (EC_{20}), 50% (EC_{50}) and 80% (EC_{80}) induction [23].

The 20–80% range encompasses the linear part of the Hill fit and it is, preferentially, this part of the curve that is used for quantification of dioxin/furan and PCB extracts. It has been reported and explained by [27] that it is better that the dioxin fraction (after an appropriate dilution of the sample extract) should fall below the 75% induction mark (whereas the PCB fraction should be below 50% TCDD induction). When quantifying dioxin or PCB fractions, most often extracts near the 50% TCDD induction range are prepared to ultimately provide a CALUX-BEQ. Even better would be to have full dose-curves (from lower to upper plateau) to take matrix effects such as non-additivity and signal depression at high concentration into account, but due to low level concentrations in sample matrices such as for example blood samples, this is not always feasible.

The EC_{20}, EC_{50} and EC_{80} points are therefore quite important and since ultimately the CALUX tool is to be used as a semi-quantitative approach to determine dioxin and PCB contents by application of the Hill equation, the three points can be used as an in-house Quality Control (QC) tool. They can be plotted in a control chart so that extreme values are readily detected and rejected.

Individual results from the Hill fit were analyzed and summarized in Table 3. From this table we can see that variation increases with higher TCDD induction (higher amount of TCDD added) and that based on the EC_{50} values, the newer 7.5 cell line is approximately 3 times more sensitive than the 6.1 cell line. This factor is somewhat lower for the EC_{20} value, somewhat higher for the EC_{80} value. This can be seen in Fig. 1 where both curves are plotted as the averaged induction. It was opted here to express values for the ECs at 20, 50 and 80% as a concentration (pM) of the treatment solution

Table 4
Within day repeatability and between day reproducibility of the 6.1 and 7.5 cell lines expressed at the 20, 50 and 80% induction mark.

	6.1 cell line		7.5 cell line	
	Repeatability S_r (CV, %)	Reproducibility S_R (CV, %)	Repeatability S_r (CV, %)	Reproducibility S_R (CV, %)
EC (20)	6.7 ($n = 16$)	8.1 ($n = 16$)	12.2 ($n = 12$)	12.2 ($n = 12$)
EC (50)	9.4 ($n = 16$)	9.9 ($n = 16$)	5.8 ($n = 12$)	5.8 ($n = 12$)
EC (80)	13.2 ($n = 16$)	13.2 ($n = 16$)	10.2 ($n = 12$)	10.2 ($n = 12$)

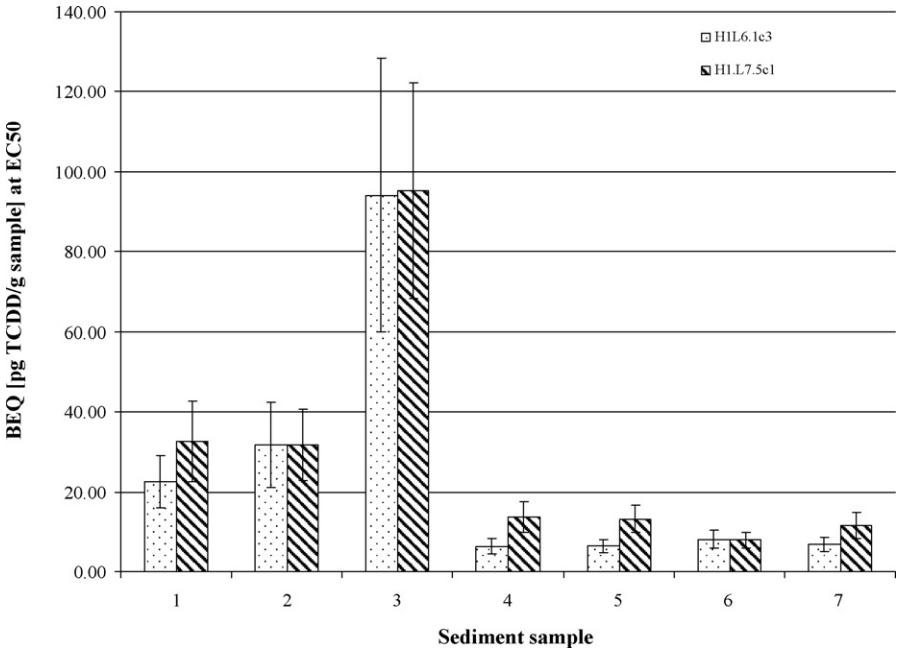

Fig. 2. BEQ-values (pg TCDD/g sample) for dioxins present in sediment samples and analyzed with both the 6.1 cell line (bars with dots) and the 7.5 cell line (bars with dashed lines). The error bars display the uncertainty on the measurement.

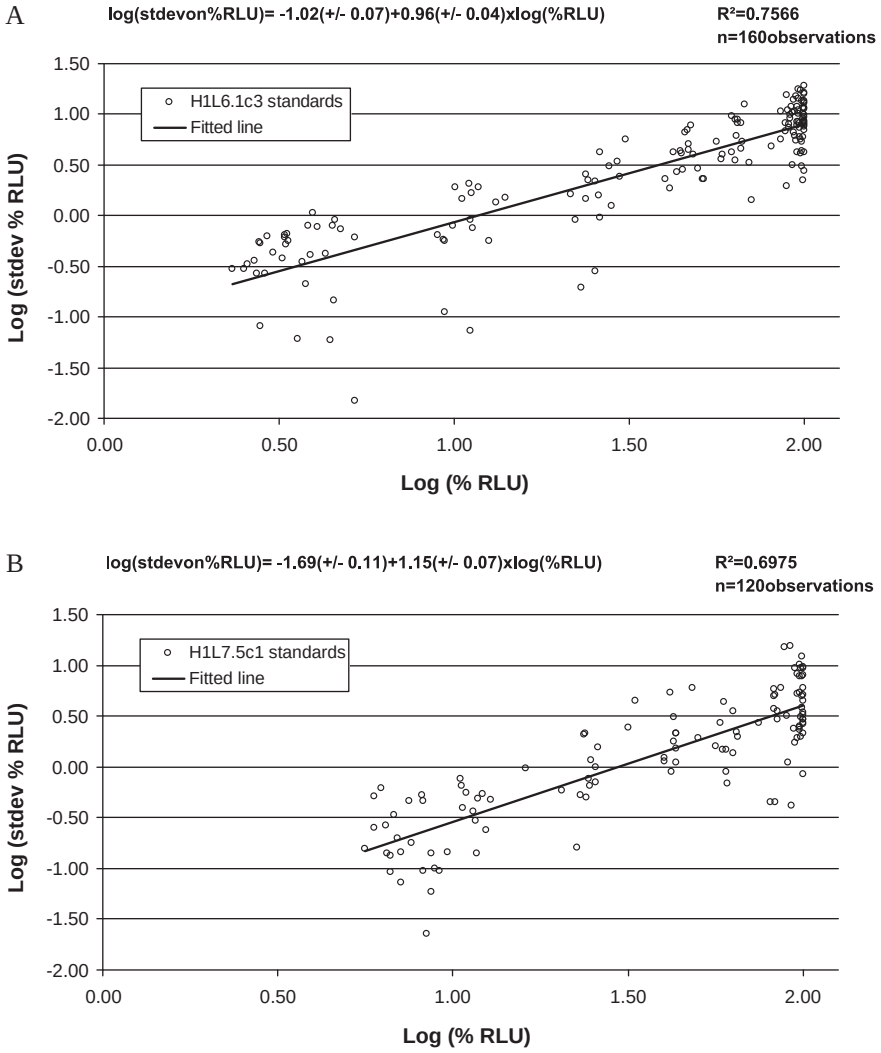

Fig. 3. Power relationships (upper equation in respective graphs) for the log (stdev on %RLU) vs. the log(%RLU), with (A) the H1L6.1c3 cell line (n = 160 observations) and (B) the H1L7.5c1 cell line (n = 120 observations).

(4 μl TCDD standard solution in 396 μl of media), with an applied dosage volume of 100 μl in one well.

For comparison reasons, the weight-based results (taking into account standard concentration, volumes used and volumes dosed as well as the molecular weight of TCDD) for the EC_{50} value are also provided here: 0.656 pg on average for the 6.1 cell line vs. 0.224 pg for the newer 7.5 cell line. The values are in accordance with literature data expressed in pM [5,15,18,28].

3.3. Sensitivity

The two lowest standards (1 and 5×10^{-11} M) yield the same response for the 6.1 cell line, while this is not the case for the other one (Table 2). This means that the 6.1 cell line can only quantify TCDD concentrations above 5×10^{-11} M, while the 7.5 cell line is able to go down to 1×10^{-11} M, which makes it in this respect about 5 times more sensitive.

Since most measurements, and especially on food and feed samples, are performed near EC_{50}, in that case the 7.5 cell line is 3.0 times more sensitive, while it is 2.2 and 3.9 times respectively at EC_{20} and EC_{80}.

3.4. Repeatability and reproducibility

Table 4 summarizes the outcome for the repeatability and reproducibility coefficients of variation, where it can be seen that coefficients of variation are below 15% but that they increase with induction in the case of the 6.1 cell line. The 7.5 cell line shows a minimum coefficient of variation at the 50% TCDD induction mark. The S_L term is generally very small in the case of the 7.5 cell line due to very low variability between days as is indicated by simply comparing the means of multiple days, which are in very close agreement with each other (data not shown here). This in fact means that more variation occurs within-day as opposed to between-day, more specifically in the case of the 7.5 cell line.

As previously reported [18], there is a functional power relationship between the precision values (standard deviation) and the response signals (RLUs). In Fig. 3, it can be seen that the slopes appear significantly different from 0, which are typical of heteroscedastic data (non-constant variance), and the R^2-values suggest that > 60% of the variance in the standard deviation are explained by the regressions. From these results, the averaged precision that is expected on TCDD replicates should at least be better than 10% and 5% for cell lines 6.1 and 7.5, respectively. These values are in good agreement with those reported elsewhere [18].

3.5. Response of both cell lines on natural samples

To test whether both cell lines produce the same result, a small number of sediment samples were analyzed with both cell lines. Samples were extracted, cleaned-up and separated into a dioxin and PCB fraction. In Fig. 2 the response for the dioxin fraction of the samples can be expressed in a BEQ manner (pg TCDD/g sediment). Dose-curves were made by serial dilution of extracts and sample BEQ_{50}s were calculated (theoretically defined as the EC_{50} value of TCDD expressed in pg divided by the EC_{50} of the sample as g sediment sample used). The sample response for BEQ calculation was also taken at 50% of TCDD induction in case the maximum of the sample dose-curve could not be attained.

From the data presented in Fig. 2 we can see that the results obtained with the 7.5 cell line are in close agreement with those of the 6.1 cell line. Similar observations could be made on human serum samples (Croes K., personal communication). The performance of the new 7.5 cell line looks sufficiently promising to apply it on much more samples and sample types in the future.

4. Conclusion and future considerations

The recently developed 7.5 cell line has definitely higher induction values than the 6.1 cell line which aids in stabilizing the signal and in providing better coefficients of variation. The 7.5 cell line also allows assessing lower concentrations than the 6.1 cell line. Most samples are quantified at the EC_{50}-value (half the maximum induction that is arbitrarily set equal to 100%): at this induction percentage the newer 7.5 cell line is 3 times more sensitive than the 6.1 cell line with respective values of 6.9 pM TCDD (0.22 pg) and 20.4 pM TCDD (0.66 pg).

A limited number of natural sediment samples were analyzed by both cell lines. The observed results were in very good agreement.

Work for the future includes the design of the plate for sample quantization; this will require the presence of a QC solution (in-house) of only TCDD, QC mix solution and possibly the inclusion of a reference sample for QA/QC purposes. This is of course dependent on the requirements of the CALUX bioassay to be a screening or a semi-quantitative method.

A larger number of samples (both in number and matrix type) will be tested in the future to generate CALUX-BEQ values that can be compared between the two cell lines.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.talanta.2011.07.042.

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