Application Note

QUANTIFYING EMERGING POLLUTANTS WITH THE CENTRO XS AND THE CALUX® PANEL FROM BIODETECTION SYSTEMS

FAST, SIMPLE, AND ACCURATE QUANTIFICATION OF DIOXINS, PFAS, EDCs AND OTHER EMERGING POLLUTANTS

Abstract

CALUX® assays are effect-based bioassays that allow the high-throughput screening of a broad range of toxic substances in water, food and feed samples. Under the conditions tested, the Centro XS was able to successfully measure all CALUX assays tested with unmatched performance.

Introduction

To ensure safety and health of the population, it is necessary to check for the presence of toxic substances in water, food, feed and the environment. Such toxicants are often quantified using targeted chemical analysis; however, there are thousands of such substances, which may be found in complex mixtures, and determining them individually is challenging or even not feasible. An alternative to targeted chemical analyses are effect-based methods: instead of quantifying a specific substance, they measure its biological effect. CALUX® bioassays are effect-based methods that allow high-throughput screening of complex mixtures of chemicals such as hormones, endocrine disrupting chemicals (EDCs), persistent organic pollutants (POPs), per/polyfluorinated compounds (PFAAs/PFTs), drug, anabolic steroids and more. In this Application Note we report the suitability of the Centro XS Microplate Luminometer to quantify CALUX® assays.

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CALUX® bioassays

A CALUX® bioassay can be used as a tool in animal-free toxicological research. In a CALUX® assay, CALUX® cell lines are used in which human cells have been modified with a luciferase reporter gene derived from a firefly. Each cell line contains, in addition to the luciferase gene, a selective and specific responsive element that can interact with the corresponding receptor of the cell line. The level of luciferase expression in the cells provides insight into the possible interaction of a substance or a complex mixture of substances with the receptor in question. In each cell line, the luciferase gene together with the responsive element is inserted at a different location in the genome, allowing different receptors to bind. This

makes it possible to investigate a substance or mixture of substances for the biological activity of different pathways in the human cell. When a substance exhibits biological activity in a cell, the ligand can bind to the receptor, after which this complex binds to the responsive element. This leads to transcription of the luciferase gene. After the addition of luciferin as a substrate for luciferase, the amount of emitted light can be measured [1].

LB 963 Centro XS Microplate Luminometer

Figure 1: the Centro XS microplate luminometer combined with the PAA S-Lab microplate handler for high throughput analysis.

The Centro XS is a high-performance, easy to use microplate luminometer that provides excellent performance and flexibility:

- Superior sensitivity (<1.8 zmol firefly luciferase)
- Negligible crosstalk (10^{-6})
- Built-in shaker
- JET-injectors
- Ergonomic design
- Automation compatibility

The Centro XS is ideally suited for all luminescent reporter gene assays, immunoassays (LIA, ILMA) and biochemical assays.

To meet your compliance requirements, a set of validation tools and optional software providing 21 CFR part 11 compliance are available.

Principle of the CALUX® assay

CALUX® cells consist of a panel of reporter gene cell lines developed from U2OS human osteosarcoma cells and H4IIE rat liver cells [1, 2]. The dioxin receptor (DR) CALUX® cell line was the first CALUX® cell line developed in H4IIE cells. The DR CALUX® is used to detect biological activity of the aryl hydrocarbon receptor (AhR, also referred to as the dioxin receptor) in, for example, water following ISO 24295 standard [3]. This receptor can be activated by dioxins or dioxin-like substances.

To develop the cell line, H4IIE rat liver cells were transfected with a gene construct containing a dioxin-responsive element (DRE) and the luciferase reporter gene (pGudLuc1.1 plasmid) and a gene construct containing the neomycin gene (pSV2-neo plasmid). G418 is used for selection of transfected cells, which is possible because of the neo gene in the pSV2-neo plasmid.

The AhR is a ligand-dependent transcription factor. When a dioxin or dioxin-like compound binds to AhR, a complex is formed in the cell nucleus with the Ah receptor nuclear translocator (ARNT), which binds to the DRE. Because the DR CALUX® cells are transfected with a DRE and a luciferase gene, luciferase is produced upon activation of the DRE. Detection of the bioactivity of the DRE and luciferase gene is performed by adding luciferin to the cells. Luciferase acts as a catalyst in a reaction with luciferin, resulting in the emission of light. The reaction between luciferin and luciferase takes place in two steps. First, luciferin and luciferase form luciferyl adenylate, consuming adenosine triphosphate (ATP). Subsequently, luciferyl adenylate is converted to oxyluciferin, during which light is emitted. The amount of emitted light is measured using a luminometer (see Figure 2).

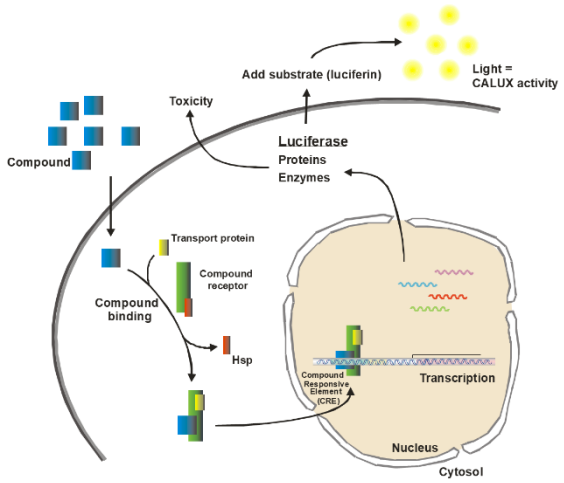

Figure 2: a schematic overview of the principle of CALUX® assays: the entering of the compound into the stable transfected CALUX® cell line, their ligand binding and transport into the nucleus. Here the compound/ligand binds to a responsive element, luciferase genes are activated and luciferase is formed. By adding luciferin reagent luminescence will be formed and analysed by luminometer

CALUX® cell lines

After the DR CALUX® cell line, many other CALUX® cell lines have been developed. These often operate according to the same principle of a receptor that, when bound to a ligand, forms a complex with the corresponding responsive element, thereby activating luciferase transcription. Most CALUX® cell lines have been developed in U2OS human osteosarcoma cells. Table 1 provides an overview of all CALUX® cell lines developed by BDS, typical chemicals tested and international standard regulations [1]. The pathways that can be detected by CALUX® cells are important pathways in cellular function. Disruption of such a pathway can, for example, cause metabolic syndrome or lead to problems in hormonal or inflammatory function. In addition, a substance can have a carcinogenic effect on a cell by activating the p53 pathway and stress and cytotoxic pathways [1].

CALUX®	Toxicity	Pollutant	Standard
ER	Estrogenicity	Hormone, biocide	OECD TG455, ISO 19040-3, DB ALM 197
AR	Androgenicity	Hormone, Plastic additives	OECD TG458, DB ALM 197
TR	Thyroid hormone	PFAS	CEN WA 18201
DR	Ah receptor	Dioxins, PCBs, PAHs	ISO/DIS 24295; EU 2017/644
Steroidogenesis H295R	Hormone production	Cosmetics	OECD TG 456

Table 1: list of CALUX® bioassays, their toxic key event, typical emerging pollutants and relevant international standards

Materials

- LB 963 Centro XS Microplate Luminometer from Berthold Technologies (Id. Nr. 70325-20).
- S-Lab™ Pro automated plate handler from PAA
- White 96- and 384-well microplates.
- Tubes of various volumes.
- Pipettes and pipette tips (various volumes).
- For a list of assays used, see table 2.

Instrument settings

- Reading mode: Luminescence Endpoint
- Counting time: 0.1 s
- All other settings with default values

Methods

Reagents were prepared following the manufacturer's instructions. Following the standard operation procedure (SOP)included with the kit, different standard curves were prepared from different CALUX bioassays

Equal volumes of kit standards and Luciferin reagent were mixed for each standard point.

The mix was incubated for 5 minutes at room temperature in the dark. 200 µL of each mix were pipetted in triplicate in the wells of a white 96-well plates. DMSO is used as blank.

The plate was inserted in the luminometer reader and measured with the settings detailed above.

Blank values were subtracted from the values of the standard. Data were exported from the software to xls format, and standard curves were drawn in Excel.

Calculations

The raw data of a CALUX assay measured with a luminometer consist of relative light units (RLU). RLU is a relative unit that represents the emitted light of a sample. Because RLU is a relative unit, these values are converted into the relative induction percentage (RI%), allowing different measurements to be

compared. The RI% is based on the EC50 or IC50 and the amount of emitted light of a sample. The EC50 is the concentration of an agonistic substance at which half of the maximal response of the CALUX assay is reached. The IC50 is the concentration of an antagonistic substance at which half of the CALUX response is suppressed.

By converting RLU to RI%, the values are no longer relative but reflect the minimum and maximum amount of light emitted by the CALUX cells. RLU values are converted to RI% by subtracting the average RLU value of the triplicate blank measurement (bottom) from the RLU values of each individual other measurement on the same (96-well) plate. The resulting values are then divided by the average of the RLU values of the two highest measured reference concentrations in triplicate and multiplied by 100.

By exposing CALUX cells to a dilution series of a reference substance, the minimum and maximum response and the EC50 can be determined by constructing an S-curve. The induction of a tested substance can thus be determined and is expressed in equivalents. To assess the reliability of a measurement, the induction factor (FI) and the Z-factor are determined. The FI is the ratio between the bottom and the RLU value of a measurement on the same plate. The result indicates how much biological activity of the added substance has been measured in the cells, without taking background signal into account. The Z-factor is the difference between the highest and the lowest measurement. A Z-factor that is too low indicates that the level of cell activity is insufficient to yield a reliable result [OECD TG 455 and TG458 standards].

The results of a CALUX assay must meet certain acceptance criteria in order to be used. These acceptance criteria were established in collaboration with the Organisation for Economic Co-operation and Development (OECD). Different CALUX assays have different acceptance criteria. A panel of CALUX bioassays used as high-through-put screening testing (HTPS) in 384 well plates is described in the ECVAM DB ALM 197 method description.

Results

A wide range of CALUX bioassays with reference compounds and their limit of quantification (LOQ) are listed in Table 2. Obtained LOQs range from $6.5 \cdot 10^{-3}$ M for the TCF receptor (reference: lithium chloride) to $2.54 \cdot 10^{-13}$ M for the Arylhydrocarbon receptor (reference: 2,3,7,8-TCCD).

Bioassay	Receptor	Reference	Mw (g/mol)	LOQ (M well)
ERα	estrogen receptor alpha	17b-estradiol	272.38	2.53E-12
anti-ERα	estrogen receptor alpha antagonism	Tamoxifen	371.51	1.33E-08
ERβ	estrogen receptor beta	17b-estradiol	272.38	2.27E-11
AR	androgen receptor	dehydrotestosteron (DHT)	290.44	1.40E-11
anti-AR	androgen receptor antagonism	Flutamide	276.21	1.05E-07
PR	progestin receptor	Org2058	344.49	2.97E-11
anti-PR	progestin receptor antagonism	Ru486	429.59	4.17E-11
GR	glucocorticoid receptor	Dexamethasone	392.46	2.22E-10
anti-GR	glucocorticoid receptor antagonism	Ru486	429.59	6.33E-10
TRβ	thyryroid receptor beta	T3 (Triiodothyronine)	650.97	1.96E-10
anti-TRβ	thyryroid receptor beta antagonism	Deoxynivalenol	296.32	2.44E-07
PPARa	Peroxisome proliferator-activated receptor alpha	GW7647	502.75	2.25E-10
PPARd	Peroxisome proliferator-activated receptor delta	L-165,041	402.44	1.06E-08
PPARg	Peroxisome proliferator-activated receptor gamma	Rosiglitazone	357.43	3.43E-09
LXR	liver X receptor	GW3965	618.51	4.26E-09
NFkB	NFkB receptor	TPA	616.83	1.27E-10
RAR	retinoid receptor	all trans-retinoic acid	300.4	5.00E-10
TCF	TCF receptor	Lithiumchloride	42.39	6.50E-03
Hif1a	Hypoxia-inducible factor 1-alpha receptor	Cobaltous chloride	129.84	1.08E-05
NRf2	Nuclear factor erythroid 2-related factor 2 receptor	Curcumin	368.38	4.77E-06
P53 (-S9)	cellular tumor antigen p53	Actinomycin D	1255.42	6.73E-10
P53 (+S9)	cellular tumor antigen p53	Cyclophosphamide	261.08	1.49E-04
P21	cyclin-dependent kinase inhibitor 1	Actinomycin D	1255.42	4.12E-10
AP1	Activator protein 1	TPA	616.83	1.89E-10
ESRE	ESRE receptor	Tunicamycin	830.92	1.81E-08
PXR	pregnane X receptor	Nicardipine	515.99	1.87E-07
AhR (DR)	Arylhydrocarbon receptor	2,3,7,8-TCDD	321.97	5.42E-13
AhR (DR#10)	Arylhydrocarbon receptor	2,3,7,8-TCDD	321.97	2.54E-13
PAH	Arylhydrocarbon receptor	B[a]P	252.31	2.49E-10

Table 2: stable transfected CALUX® bioassays, their receptor-ligand binding and standard reference compounds (incl. Molecular weight Mw) with typical limit of quantifications (LOQ) by using Centro XS luminometer readout.

Discussion

Quantification of luminescence is very often performed using a microplate luminometer. By using the Centro XS series, a high throughput with 96-well plates and a lowest sensitivity of all so far at BDS tested luminometer are guaranteed. By using the PAA S-Lab stacker, a decrease in costs and preparation time has been validated and approved. The limit of detection obtained with the Centro XS instrument is clearly better than the typical result for other microplate readers, and the following luminometer specifications for CALUX BIOASSAYS have been validated and proven:

1. Maximum measurement sensitivity of <1.5 amol ATP and <1.5 zmol Firefly Luciferase. Largest dynamic measuring range of 6.5 decades through the use of selected, low-noise photomultipliers that are operated in a digital single-photon-counting mode is required.
2. Low background of typically < 10 RLU and lowest possible crosstalk of < 10^{-6} due to specialized sample chamber and measuring head arrangement.
3. Up to 2 variably adjustable in volume and mixing speed reagent injectors with the highest precision thanks to stepper motor-controlled bellows technology and adjustable jet injection for rapid and complete mixing of the reagents, arranged outside the measuring position for safety reasons, can be repeated as often as required in the respective measuring cycles for kinetics and repeat measurements. Compared to syringe injectors - with us complete emptying, no mixing and significantly lower dead volume.
4. Injectors are built directly into the device to save space and are easily accessible, no additional space is required, and the supply of reagents is clearly visible during operation.

5. Additional automatic waste pump, which means no extra empty plate is required for washing and priming. Refreshing and pumping back of reagents into the storage vessels is possible.

Conclusions

These results demonstrate the high performance of the Centro XS Microplate Luminometer for the quantification of luminescence using stable transfected CALUX® cell lines. The system not only enables compounds concentration measurements (e.g. dioxins, PFAS, BPA, PAHs) over a wide dynamic range but can also helps increase the throughput of this qualification step in important applications such as for compounds testing (e.g. cosmetics, pharmaceuticals, PFAS) and their complex matrices (e.g. water, food, plasma).

References

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