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# Catalogue

***BioDetection Systems BV***  
Your bio-analytical service partner



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## List of abbreviations

AhR	aryl hydrocarbon receptor
AP1	activator protein 1
AR	androgen receptor
BEQ	bioanalytical equivalent
CALUX	chemically activated luciferase activity
DB-ALM	database of alternative methods
DMSO	dimethylsulfoxide
DR	dioxin-responsive
EC50 (10, 20, etc.)	50(10, 20, etc.)% effect concentration
ER	estrogen receptor
ESRE	endoplasmic reticulum stress response element
EURL-ECVAM	European Reference Laboratory-European Center for Validation of Alternative Methods
FCS	fetal calf serum
GC-MS	gas chromatography-mass spectroscopy
GR	glucocorticoid receptor
HIF1alpha	hypoxia-inducible factor subunit 1alpha
IATA	integrated approach to testing and assessment
ISO	International Organization for Standardization
LOQ	level of quantification
LXR	liver X receptor
NAM	new approach methods
Nrf2	nuclear factor erythroid 2–related factor 2
OECD	Organisation for Economic Cooperation and Development
PC50 (10,20, etc.)	50(10, 20, etc.)% effect concentration related to a positive control
PAH	polycyclic aromatic hydrocarbons
PCB	polychlorinated biphenyls
PFAS	poly-and perfluoroalkyl substances
PPAR	peroxysome proliferator activated receptor
PR	progesterone receptor
PXR	pregnane X receptor
QA/QC	Quality assurance and quality control
RAR	retinoic acid receptor
S9	liver extract
SME	Small or medium enterprise
SPE	solid phase extraction
TCF	T-cell factor
TEQ	toxic equivalent
TG	test guideline
TR	thyroid receptor
TTR	transthyretin

**Dear Customer,**

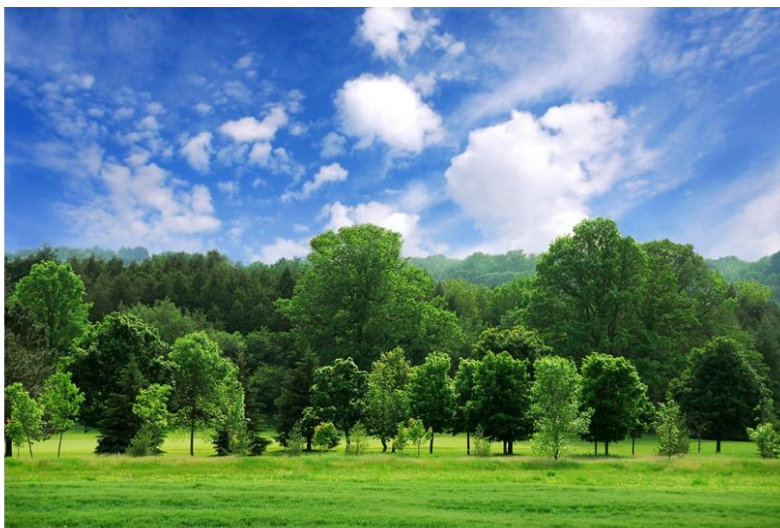
At BDS we are proud to have a long standing to work together with companies, knowledge- and academic institutes worldwide, providing them with high quality products and experienced technical customer support.

Under our CALUX® brand we offer a broad range of cell- and effect-based bioanalysis tools as well as optimized cell culture media, standards, and reference materials. BDS provides its services through its own service laboratory, and under license by laboratories across the globe.

The current catalogue provides you with an overview of the current possibilities. However, being an innovative company, we would be pleased to discuss any other options to work with you and provide you with specific expertise or tools.

We are looking forward to work with you in protecting health and the environment!

*Your BDS Team*



## About BioDetection Systems

BDS is specialized in mechanism-based biological monitoring to protect human health and the environment. As such BDS offers an extensive panel of high-throughput-compatible CALUX® reporter assays and complementary technologies. This enables hazard identification and effect-based compound and compound mixture detection. The assay panel can be used to rapidly evaluate major types of toxicity relevant for regulatory risk assessment, allowing assurance of consumer safety and -confidence. Quantitative measurements allow assessment of points-of -departure in chemical risk assessment, replacing animal experimentation. Specific work-up methods coupled to quantitative measurements allow precise mixture effect assessment of specific chemical classes. Methods have been extensively and independently validated and are included in relevant international guidelines. Since our assays target key pathways in cells and human physiology, we increasingly employ these methods in the discovery of compounds and compound mixtures with beneficial health effects. BDS provides these services through its own service laboratory, and under license by laboratories across the globe.

*Please enquire for our accredited services, licensing, or contract research possibilities*



## Services



The BDS service laboratory offers a variety of biological and chemical analytical techniques for safety and bioactivity assessment in a wide range of areas, according to standardized operational procedures. In many cases the analysis reports already fulfil the clients' needs. For more complex projects our experienced staff, which comprises (molecular) biologists, chemists, and toxicologists, may be involved in data interpretation, consultancy and project management. In addition to that, our research experts and facilities are available for contract research and validation studies.

## Analytical services

### Effect-based bioanalysis

BDS has generated a panel of CALUX<sup>®</sup> cell lines that respond to chemically induced alterations of key cellular pathways (see Product section for details). Exposure of the respective cells to the chemicals class of interest results in the production of luciferase and the emission of light. This signal predicts the type and magnitude of the biological effect that can be expected from exposure to the chemical or chemical mixture. We employ these methods in the discovery of compounds and compound mixtures with beneficial health effects, like functional foods, and drug candidates. When, however, these pathways are overstimulated or repressed, effects of chemicals may become adverse, which forms the basis of our effect-based safety assessment. CALUX assays are used to quantitatively assess hazard profiles of chemicals and complex mixtures. In addition, assays have been developed for targeted analysis of groups of chemicals addressing the same biological effect, such as dioxins and PFASs. Analyses carried out in our service department are strictly confidential and are performed under ISO17025 and GMP+ certified conditions. BDS offers its technologies and services world-wide through a network of laboratories and agents.

#### *Benefits of BDS' CALUX<sup>®</sup> technology for safety- and bio-activity assessment*

- ✓ High throughput panel covering major health effects
- ✓ Sensitive and specific
- ✓ Easy sample clean-up / work-up
- ✓ Applicable to a wide variety of matrices and many applications
- ✓ Accepted in international legislation and guidelines, e.g., EU, OECD, ISO
- ✓ Offered under ISO17025/GMP+ certified conditions
- ✓ Complementary assays and analysis to match your application
- ✓ Short turn-around time
- ✓ Cost-effective
- ✓ Professional services, backup, and consultancy
- ✓ Global sales and support network

## Safety assessment

A wide range of assays is used by BDS to assess hazard profiles of chemicals and complex mixtures. Their application is in safety assessment and quality control of food, chemicals, cosmetics and pharmaceuticals, bio-based materials, water and the environment, for bioactivity profiling of clinical samples and functional foods, and any other application requiring straight forward animal-free safety assessment. These measurements are relevant to predict toxic effects like genotoxicity/carcinogenicity, endocrine disruption, reproductive toxicity, and interferences with lipid metabolism/obesogens.

### Genotoxicity/carcinogenicity

Our p53 CALUX® assay detects compounds that are genotoxic by causing damage to the DNA. This assay can optionally be performed in combination with microsomal S9 fractions to detect compounds that require metabolic conversion to be active. In addition, our assay panel includes a wide range of assays involved in proliferation and differentiation steps that are relevant for non-genotoxic carcinogens, including several nuclear receptor- and dioxin-receptor pathway assays.

### Endocrine disruption

Endocrine disruption has been recognized as a priority endpoint in safety evaluation of chemicals and consumer products. Endocrine disrupting compounds (EDCs) are being classified as substances of very high concern and regulations to restrict their use are being installed world-wide. EDC effects are hard to predict and are best tested using in vitro bioassays. To avoid animal studies BDS has developed a range of assays that allow screening and safety assessment of chemicals and consumer products in a rapid and cost-effective manner:

- Assessment of interferences with estrogen-, androgen- and thyroid pathways and steroidogenesis (EATS) using robust and quantitative human CALUX reporter gene technology, and complementary assays
- Standard operation procedures for extraction, clean-up and analysis to measure in a wide variety of products and applications
- Used world-wide in major chemical-, pharmaceutical-, cosmetics-, food- and feed companies, and others
- Extensively validated to demonstrate robustness and predictivity

### ***Our EATS endocrine disruption panel***

- **E:** (anti)Estrogens: ERalpha CALUX (OECD TG455)
- **A:** (anti)Androgens: AR CALUX (ECVAM validation, OECD TG458)
- **T:** Thyroid interference: (anti)TRbeta CALUX, and TTR assay (TG in preparation)
- **S:** H295R steroidogenesis (OECD 456)
- ***Phase 1 and 2 metabolic steps***



### Reproductive toxicity

Reproductive toxicity, including developmental toxicity involves a large variety of possible mechanisms and endpoints. Precise predictions of these effects is challenging, also when using the golden standard cost-, animal- and labour intensive animal tests. Concordance between these regulatory animal tests in different species is known to be around 60% only, not very far away from flipping a coin. Therefore, alternatives are urgently needed. Remarkably, when evaluated in a battery of state-of-the art relevant *in vitro* tests, the CALUX® high throughput battery performed equally well as the entire battery, clearly demonstrating its applicability in this important area.

### Interferences with lipid metabolism/obesogens

This CALUX® panel contains assays that can measure the activity of all three members of the peroxisome proliferator activated receptors (PPARs). These are linked to beneficial health effects, but when aberrantly stimulated may also lead to adverse effects. In particular, concerns have been raised that some environmental compounds act as so-called obesogens by targeting PPARs. This opens another application area of the PPAR assays, and tests installed to further validate when possible adverse effects are found.

### Other biological effects

In addition to the exemplified effect-based assays and their applications, we have a wide range of specific CALUX® assays for other major mechanistic pathways, including chemical stress-induced protective- and detoxification pathways. These are involved in a wide range of classical toxicological endpoints as assessed in animal studies, like acute toxicity and sensitisation. When overstimulated effects will become adverse, corresponding to the so-called adverse outcome pathways (AOPs). Please enquire for your specific effect class of interest.

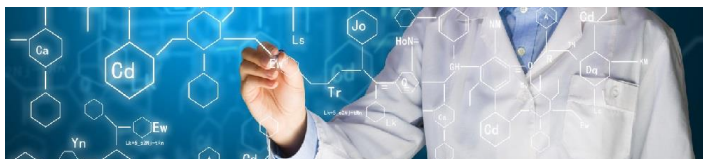
### Beneficial health effects

The CALUX® reporter gene assays address very specific effects pathways, facilitating measurement and interpretation of results. The pathways included are central in regulating cell functioning and are interesting targets for drugs and nutraceuticals. This includes ones that are relevant for obesity and metabolic syndrome (e.g. PPARs, dioxin- and steroid receptors), endocrine functioning (pathways e.g. related to sex steroid receptors), (anti)inflammatory and immune modulatory pathways (e.g. glucocorticoid receptors, NFkappaB signalling, sex steroid receptors and dioxin receptor), cancer (p21, p53 pathway, stress- and cytotoxicity pathways, angiogenesis, retinoid receptors, steroid receptors), and antioxidants/chemoprevention (nrf-2, retinoids), and others. With this high throughput screening (HTS) panel activities of interest can be rapidly identified in natural and environmental samples and in compound libraries for screening potential drug candidates. Additional testing to validate these hits can be carried out in more physiological models. Furthermore, an assessment of stability and the effect of metabolic conversions can be made. In combination with data on traditional use of the starting material, promising leads for improved formulations or new drug candidates can be identified.

Securing your safety by  
non-animal testing



## Targeted (bio)analysis



Humans typically are exposed to complex mixtures of both natural and synthetic chemicals. Effect-based bioanalysis provides the most comprehensive view on potential hazards or benefits of these complex mixtures. However, regulations mostly still require targeted analysis of specific compounds. BDS has more than 20 years of experience bridging these requirements by using chemical compound class selective quantitative bioanalysis in combination with selective extraction and workup methods. Our service laboratory is ISO 17025 (registration number RvA L401) accredited for analysis of dioxins, furans, and dioxin-like compounds, non-dioxin-like PCBs and estrogenic compounds, while new methods are being developed. In addition to quantitative and selective bioanalysis, state-of-the-art chemical analytics is offered.

### Dioxins, furans and dioxin-like compounds

Chemical safety is a high priority issue for the food and feed sector as it directly impacts human and animal health. Stringent EU limit values are in force for dioxins in food- and feeding stuffs for animal and public health protection (Commission Regulation (EU) No 589/2014 (food) and Commission Regulation (EU) No 278/2012 (feed)). The use of the DR CALUX<sup>®</sup> bioassay for monitoring dioxins in food and feed allows the (pre)-selection of samples suspected of being contaminated above regulatory limit values with dioxins. Dioxins and dioxin-like compound analyses by the aryl hydrocarbon receptor (AhR) based, DR CALUX analysis method in various food and feed matrices is ISO 17025 accredited (RvA L401), while the California State Water Board has approved BDS for AhR based analysis of dioxins in (recycled) water.

### Polychlorinated biphenyls (PCBs)

PCBs are a highly toxic class of persistent industrial chemicals that are often addressed as a compound class in safety assessments. PCBs can be divided in two groups: the dioxin-like PCBs (dl-PCBs) and the non-dioxin-like PCBs (ndl-PCBs). Dioxin-like PCBs show toxicological effects similar to those of dioxins and can be measured with the DR CALUX<sup>®</sup> analysis method. Non-dioxin-like PCBs are also regulated for food and feed samples according to the above-mentioned EU regulations. In contrast to dioxins and dioxin-like chemicals, non-dioxin-like PCBs are analysed using chemical-analytical methods (GC-MS/MS). BDS is also ISO 17025 accredited (RvA L401) for this method.

### Polycyclic Aromatic Hydrocarbons (PAHs)

PAHs are typically present in matrices as complex mixtures. Analytical measurements generally focus on a very limited set of the thousands of PAHs that are known to exist. The PAH CALUX<sup>®</sup> method has the advantage that it detects the toxic, carcinogenic PAHs and is therefore perfectly suitable for hazard identification.

### Poly- and perfluoroalkyl substances (PFAS)

PFAS are known to be able to interfere in thyroid hormone metabolism by e.g., disrupting the transport of the natural ligand thyroxine (T4) by a major transporter protein transthyretin (TTR). The PFAS CALUX<sup>®</sup> assay uses this important property to measure the effect of the range of PFAS compounds in chemical mixtures, independent of prior knowledge of their structure, has been shown in already. Disruption of TTR binding by T4 is benchmarked against the reference compound PFOA and expressed as pg PFOA equivalents/g sample processed. Simple sample processing and clean-up is required, allowing successful application to a wide range of matrices.

### Steroids

Endogenous steroids are key in the regulation of a wide range of physiological processes. Steroids are widely used as drugs and illegally, e.g. as growth- and performance promoters. In addition, steroid residues are found as contaminants in food and the environment. We have generated a panel of assays to measure bioactive steroids. Particular care was taken to avoid cross reactions, leading to a highly selective assay panel of estrogens, androgens, progestins and glucocorticoids that has shown its value in a wide range of applications.

### Environmental estrogens

Environmental estrogens are contaminants that can mimic the biological effects of the steroid hormone estrogen and when present at levels above established trigger values can disrupt normal estrogen action, thereby acting as so-called endocrine disrupting compounds. The detection and quantification of estrogenic compounds in e.g., (environmental) water samples by CALUX<sup>®</sup> analysis is ISO 17025 accredited (RvA L401), while the California State Water Board has approved BDS for ERalpha based analysis of estrogens in (recycled) water.

### Fractionation techniques

Many samples, e.g. from food, feed or environmental origin, contain a complex mixture of bioactive compounds. To reduce complexity and / or irrelevant background activities, BDS applies and develops chemical analytical fractionation techniques. These include solid-phase extraction (SPE), gel permeation chromatography (GPC), and high-performance liquid chromatography (HPLC). While many protocols can readily be performed in our laboratories, dedicated protocols for new or uncommon matrices may be developed on demand. In addition, BDS is experienced in identifying compounds responsible for bioactivity in complex mixtures through effect-directed analysis (EDA). For this, the fractionation techniques are coupled to bioanalysis to identify bioactive fractions. When sufficiently pure, the compounds in these fractions can be analysed on their chemical identity.

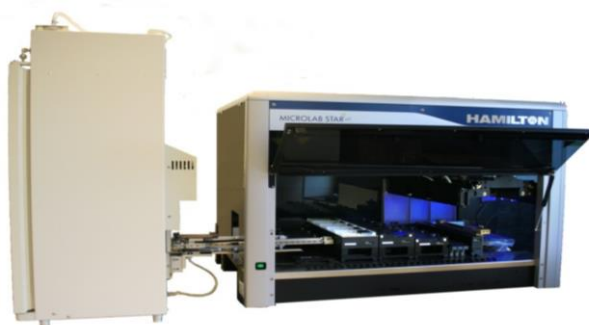


## Contract research



The combined expertise of our professionals and our well-equipped laboratories allow for a successful execution of contract research projects. These may include any combination of our skills including routine analyses of samples in our service department, development and application of novel (bio)analytical methods and the execution of complex R&D projects guided by our academically trained staff.

Our ample experience in promoting regulatory acceptance of bioanalytical tools and our extensive quality control schemes make us an attractive partner for setting up, guiding, and executing validation studies. Our contract research projects are always carried out flexibly, according to customer needs, while keeping strictly within the predetermined budgetary and time constraints.



## Consultancy



Our services focus on delivery of highly standardized and reliable analytics according to the latest guidelines. Upon request we provide professional help with additional questions these analyses may raise, for example regarding regulatory or legal compliance. With many personnel having academic training and a long-standing experience in (bio)analytics and related fields and applications we can provide solutions to complex issues independently, or together with our customer's experts.

## Accreditation and certification

Analysis quality and customer service are very important to us. Therefore, the quality of our (bio-) analytical services is assured through an internal quality system including internal audits by our quality officer, and external auditing. BDS is ISO/IEC 17025 (registration number RvA L401) accredited for bioanalysis of dioxins and dioxin-like compounds (DR CALUX) and chemical analysis of dioxins, dioxin-like PCBs and non-dioxin-like PCBs (GC-MS/MS in various food and feed matrices. In addition, we are accredited for the analysis of (pseudo-) estrogens in water samples (e.g. influent, effluent, surface water). In addition to the ISO/IEC 17025 accreditation, we have proved to meet the B10 standard of the GMP+ certification scheme as part of the GMP+ Feed Safety Assurance of GMP+ International, as well as the standards for Q&S and KAT. Most recently, we have acquired approval from the California State Water Board for analysis of AhR ligands and estrogens in recycled water using our DR-CALUX and ERalpha CALUX methodologies.

## Products



## Bioassays

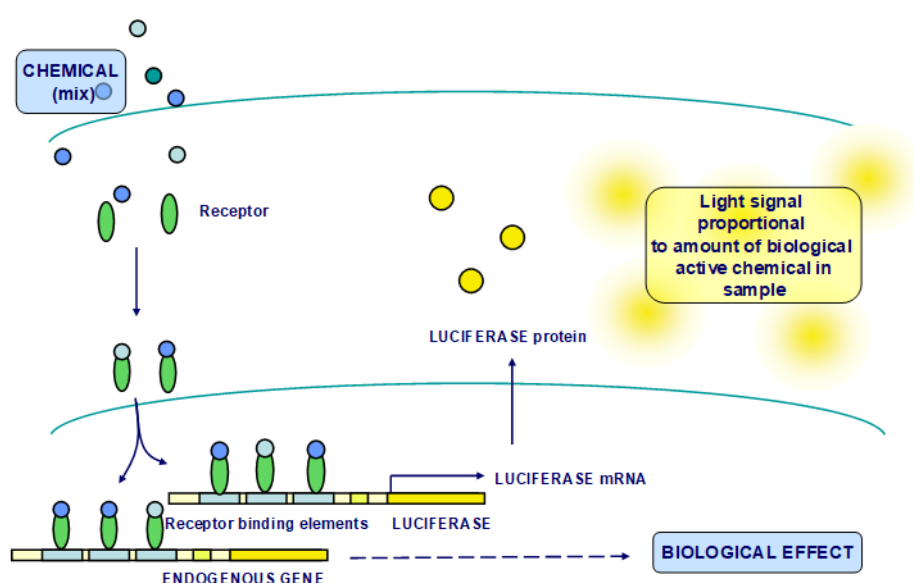
BDS offers an extensive panel of high-throughput-compatible CALUX<sup>®</sup> reporter assays that enable biological effect identification and quantification and effect-based compound detection. Our assays allow for the sensitive and precise detection of specific biological effects at the cellular level. This can be used for the identification of fractions and compounds with beneficial health effects. However, context-dependent repression or overstimulation of these pathways form the key to toxic effects of chemicals. For this, context-dependent thresholds are defined. In this way this panel can be used to predict major chemically-induced toxic effects such as sensitization, carcinogenesis and mutagenicity and, reproductive toxicity and endocrine disruption, related to health effects like birth- and fertility defects, cancer, metabolic syndrome and obesity.





## Assay principle

Mammalian cells possess a wide range of mechanisms with which they can specifically respond to signals or stresses induced by compounds they are confronted with. CALUX® (Chemically Activated LUCiferase eXpression) assays form a panel of mammalian cell lines that were modified to produce a quantifiable response in addition to this natural response: If a cell is triggered to generate a response, activation of a reporter gene leads to the production of an enzyme (luciferase) that produces light during a reaction it catalyses. This light signal is proportional to the amount of biological active chemicals in the sample. The molecular design of the CALUX assays ensures that the observed response is highly specific for the effect of interest.



## Assay procedure

CALUX® assays may be used for testing of single compounds as well as complex mixtures. The cells are cultured in well plates (usually 96 or 384 wells), which allows for increased throughput and automated handling. Subsequently, they are exposed to a dilution series of the test compound or a dilution series of an extract of the test sample. Along with the test samples, the cells are also exposed to a concentration series of a reference compound. After a certain exposure time, the light production in the individual wells is quantified using a luminometer. The activity evoked by the test compound or test sample is subsequently derived by interpolation in the response curve of the reference compound and is expressed in equivalences of this reference compound.

## Available assays

In addition to the CALUX® assays that are currently available, BDS is constantly working on new reporter cell lines and new applications with these cell lines, as well as complementary technologies for relevant applications. A complete overview is presented in the “Products” and “CALUX® reporter cell line overview” chapters.



***Please contact us to discuss different possibilities to use our assays or services***



## Various products

Matrix reference materials are included in the analysis as a quality control for extraction efficiency or performance of the assay. The matrix reference materials can also be applied for international standards, such as ISO 17025 or for the correction of the bioassay apparent recovery as required by the EU guidelines. Matrix reference materials are essential in the assessment of the accuracy and reliability of laboratory measurement processes. Our products are stable, homogeneous, well-characterized and are available for matrices such as e.g. feeding stuff, fish-oils, and egg powder. Certified values are determined by repetitive analysis in one or more analytical laboratories. On request, we can produce new materials for specific purposes.

## Standards

### Reference compounds

The results of CALUX® assays are typically expressed relative to the activity of a reference compound. We offer concentration series of reference compounds for the CALUX assays, which can be used directly.

### Calibration standards

BDS provides calibration standards for the CALUX® assays that can be used for performance control during trainings on the assay procedure, or for quality control of routine analyses with the CALUX bioassays. These standards usually contain a mixture of compounds that evoke a known response on the CALUX cells.

## Tissue culture products

We offer several products used for culturing the CALUX® cells and performing of the assays, such as trypsin, lysis mix, measuring solutions, FCS and stripped FCS, S9-metabolic enzyme mixtures. Most products we offer are CALUX-tested to ensure low backgrounds and correct functioning in combination with our assays.

## Extraction & clean-up products

BDS offers a variety of products that have been optimized for the use together with our CALUX bioassays. SPE-based water extraction products; protein-binding inhibition (TTR) columns. Our clean-up columns are optimized for CALUX analysis of dioxins are tailor-made to ensure fast-running, effective clean up with high recovery, easy cleaning ability and robustness. The columns can be delivered along with a robust placeholder both for columns as well as for collection vials for e.g. human samples.

## Training & Licensing



Our CALUX® analyses are not only offered as a service by our own laboratories, but are also performed under license in numerous other research and analysis facilities around the world.

We facilitate a successful introduction of the assay in the licensee's laboratory by offering an individual training at our facilities in Amsterdam, a follow-up coaching at the licensee's facilities and a final cross-validation study to certify the licensee's laboratory. Our trainings can be customized upon request.

***Please contact us to discuss the best options for your organization***



## Applications



Our assays and techniques are used standard service mode and suitable for numerous applications in safety assessment of our food, water, environment and products, as well as in research projects aimed at developing and improving in vitro alternatives for animal testing; methodology to better achieve high safety and quality standards and technologies; discovery of novel bioactivities and fostering applications in biobased and circular economy. Our product and application specialists are available for discussing the use of our tools and services for your specific application

## Safety

### Toxic potency of complex chemical mixtures in diet, environment and products

A limited number of industrial chemicals is tested for their potential toxic properties, while even less is known about the vast number of natural chemicals. Nevertheless, we are mostly exposed to complex mixtures of all of those compounds. Biological effects by mixtures are typically hard to predict from data on the limited number of well-characterized chemicals, since these cover only a part of the compounds actually present. Direct measurement of the total potency of toxic, or bioactive components in the mixture using CALUX® reporter assays solves these issues in the following ways:

- ✓ CALUX assays directly measure the combined effects by the total mixture that is analysed. Results are therefore independent of prior toxicological knowledge on the individual constituents of a mixture.
- ✓ Specificity of the assays and extensive quality controls avoid assay interferences that may typically occur in less well defined and validated assays.
- ✓ If desired, a combination of chemical analytical and biology-based techniques can be used to identify whether observed effects can be attributed to specific compounds or fractions.
- ✓ BDS works together with key stakeholders to incorporate assays in relevant legislation, including the definition of standard operational procedures and thresholds of adversity.

In the next sections several important applications of CALUX methods are described in brief.

#### Dioxins & PAHs in Food & Feed

BDS offers analyses that specifically target dioxins, dioxin-like PCBs, non-dioxin-like PCBs, PAHs and PFASs. In addition to the targeted screening for these toxic compounds, CALUX® assays are also used in screenings for potentially adverse biological activities, like endocrine- or genotoxic effects. The presence of dioxins and PCBs in food and feed is subjected to international regulations. The EU has

laid down general requirements for the determination of dioxins and dioxin-like PCBs in food and feed using cell-based bioassays. The DR CALUX bioassay (dioxins / furans and dioxine-like-PCBs) fully complies with these stringent requirements and is ISO 17025 accredited (registration number RvA L401). For samples that are suspected based on the DR CALUX results, we offer confirmative analysis by GC-MS/MS. Analysis of non dioxin-like PCBs is performed by chemical analysis (GC-MS/MS) only; ISO 17025 (RvA L401) accredited).

### Packaging & Consumer Product Safety

Our health may not only be affected by chemical contaminants present in our food- or environment, but also by compounds that contaminate consumer products and/or food packaging materials, and may migrate under specific conditions. Examples of consumer product-related chemicals that are under suspicion for their potential health effects are certain plasticizers, PFAS, brominated dioxins and fire-retardants. CALUX® assays can be used to control and monitor consumer safety e.g. in combination with migration studies for monitoring release of bioactive compounds from a packaging material, or for consumer product safety testing, with a focus on endocrine activity, geno- and cytotoxicity related effects.

### Water quality

Current water quality monitoring practices mainly rely on targeted chemical analysis of a selected set of compounds (e.g. priority compounds). This compound-oriented approach, however, does not cover the entire load of pollution and may overlook the presence of the vast majority of biologically active chemicals. Effect-oriented testing approaches can overcome this limitation by measuring the effect of the total mixture of compounds that are present in a sample. The CALUX® assay panel is particularly suitable for this purpose since it detects several key adverse effects in relation to water quality. Specific trigger values have been defined allowing routine monitoring of water quality and water processing steps.

### Environment

BDS offers various tests that specifically target important groups of environmental health-damaging pollutants, such as dioxins, PCBs, PAHs and PFASs. In addition, various assays are available for the screening of environmental samples on effects relating to adverse health effects such as endocrine disruption, genotoxicity and reproductive toxicity. CALUX® reporter assays are suitable for a wide range of applications regarding environmental safety assessment. Our assays have been used for identifying pollution hotspots, profiling health hazard related potencies on polluted sites, effect-directed identification of pollutants, ecological distribution of pollutants and the identification of hazards by emerging compounds. CALUX analysis is compatible with a range of environmental sampling strategies. Advantages of using CALUX bioassays in environmental screenings is that the measurement of effects directly relate to human health and very often to that of other higher vertebrates.

### Cosmetic formulations

The EU cosmetics regulation prohibits testing of cosmetic formulations on animals. To assure safety of new formulations non-animal tests like CALUX®, with their proven robustness and applicability to complex matrices, therefore are of key importance. BDS has been involved in several successful initiatives together with key industries and OECD to validate and demonstrate the applicability of such tests for cosmetics testing OECD approval was obtained for the (anti)estrogens test with ERalpha CALUX (TG-455) and for (anti)androgens with AR CALUX (TG 458), while the (anti)thyroid CALUX assay panel is under OECD review. Focus of in vitro testing of cosmetic formulations is on endocrine disruptors, via EATS testing.

### Bio-based & circular economy applications

With shrinking resources of fossil fuels for production of energy and chemicals alternatives are needed, one of which is the use of biomass. The use of biomass provides interesting options to generate a circular economy in which waste is non-existent since it is reused, following a cradle-to-cradle principle. Future biorefineries can be used to optimally use biomass in value chains with various food- and non-food products. BDS designs methods to identify novel high value biobased specialty products, while assuring safety. Safe disposal of end-of-life products and residues, allowing agricultural or other applications is essential to allow closure of the circular process. In addition, Green Chemistry principles need to be rigorously included already in the design of new chemicals and processes. BDS provides bio-based solutions to identify new bioactivities, and to assure safety and quality control to meet market requirements.

### Human health impact assessment

Human health can be affected by exposure to a wide variety of chemicals both of synthetic and natural origin (i.e. the exposome). Only when present at sufficiently high levels this may lead to adverse health effects. Classical targeted approaches measuring a relatively small set of these chemicals are very limited in their scope. By focusing on central processes that govern cellular functioning and homeostasis, CALUX<sup>®</sup> reporter assays can be used to integrate the combined effects of the exposome and relate these to adverse health effects such as endocrine disruption, obesity and metabolic diseases, carcinogenesis and reproductive- and developmental toxicity. The sensitivity and high-throughput compatibility of these assays makes them suitable both for mechanistic studies as well as screenings of large sample panels. In many cases, epidemiological studies and monitoring of human health effects are hampered by the limited amount of available sample material. In addition, many analytical techniques are costly and time-consuming to perform. High-throughput CALUX bioassays provide solutions to all these issues. Another important benefit of using CALUX assays is that they often give mechanistic insight in health effects that may otherwise only be correlated to a certain exposure from a statistical point of view. We are experienced to analyse a wide range of human tissues, such as blood plasma, breast milk, and urine.



## Non-animal safety assessment of single chemicals, active ingredients



Chemicals and pharmaceuticals are indispensable in modern daily life. Correct safety evaluation of chemicals is essential to protect users. The use of experimental animals in chemical safety evaluation is under pressure, not only because of ethical reasons, but also because these tests are expensive, time consuming, low in throughput and often give unsatisfactory predictions of human toxicity. For these reasons, we have limited toxicological knowledge of many chemicals. Our modern mechanistic CALUX® bioassays provide insight in the modes of action of toxicants and can be used as alternative methods to assess chemical safety, particularly when coupled to so-called adverse outcome pathways. These methods are particularly suited for use in integrated testing and read across procedures as described by ECHA and OECD.

### Chemicals & pharmaceuticals

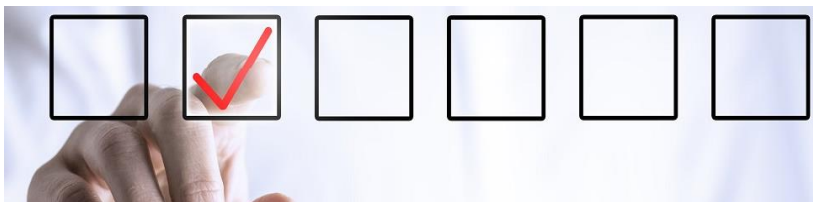
Chemical safety assessment is important to assure consumer safety and confidence. Delays in testing and evaluation of industrial chemicals are being addressed using innovative risk assessment approaches that include novel approach methods (NAMs) that are independent of animal experimentation. To enhance confidence and reduce uncertainties integrated testing methods that use all available data, including data on structurally related chemicals using so-called read-across methods are increasingly used. Mechanistic understanding is important when applying these integrated testing methods. Our specific mechanistic in vitro CALUX bioassays provide precise insight in the modes of action of toxicants and have been proven to be powerful methods in such NAM-based risk assessment, as exemplified by several case studies, including two OECD case studies on Integrated Approaches for Testing and Assessment (IATA). Major additional applications for chemical-and pharmaceutical safety assessment include screening and bioactivity profiling to select new candidate drugs and chemicals with promising profiles with respect to safety using a safe design/green chemistry approach, profiting from the high throughput of our testing batteries. All tests can be carried out using metabolising systems and methods to assess pharmacokinetics. In these evaluations additional elements like physico-chemical properties, intended application and exposure scenario, and structural similarities and alerts can also be taken into account in order to further improve predictions. With our extensive knowledge in this area, we can provide independent consultancy, while we also are happy to work with your safety officers and risk assessors.

### Cosmetic ingredients

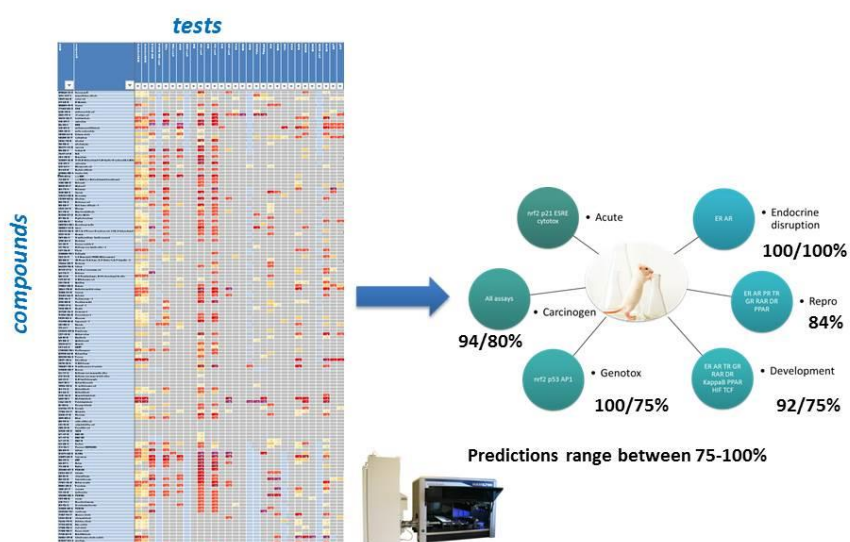
The EU cosmetics regulation prohibits testing of cosmetic ingredients on animals. To assure safety of new ingredients non-animal tests like CALUX® therefore are of key importance to assure innovation in the industry. BDS has been involved in several important initiatives together with key industries and OECD to validate and demonstrate the applicability of such tests for cosmetics testing.



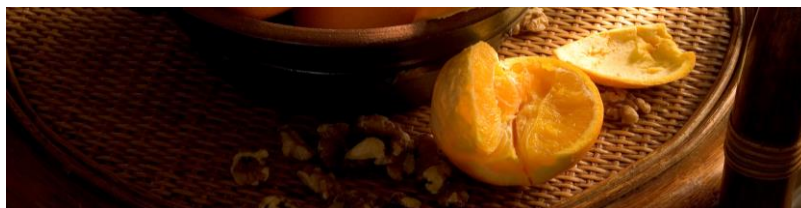
## Validation and guidelines



Good predictivity of in vivo effects is essential for successful application of an alternative testing battery. BDS spends a considerable amount of effort in quality control of its assays, validation and incorporation in international guidelines. OECD and ISO guidelines are available and BDS is actively engaged in promoting further regulatory acceptance of these novel approaches, which are generally considered as the way forward in safety evaluation. A wide range of references is available to support the excellent performance of our assays.



## Health beneficial activity screening



Our bioanalytical methods assess interference with cellular pathways that govern normal cell growth and differentiation. Some chemicals can interfere with these pathways. If this interference is strong and in an inappropriate uncontrolled context, like environmental or occupational exposure, this is unwanted and the basis of toxicity assessment. The main difference between health beneficial and toxic compounds is that the former compounds interact with these pathways in a more subtle manner, at lower therapeutic rather than toxic doses. Thus, these pathways often are equally important in the discovery of health beneficial compounds. An important asset of a druggable molecule is that it can enter the body and reach its target. Most CALUX assays are targeted by molecules with druggable properties, and by assessing entry into cells and major metabolic conversions already information on important barriers is obtained.

## Bioactive mixtures screening

Using our quantitative high throughput CALUX<sup>®</sup> panel we can rapidly evaluate the bioactivity profile of natural compound mixtures in samples of interest such as plants, fungi and biomass. Depending on the desired application, we can select the assay panel, and if needed the extraction method in such a way that we focus on the desired activity. If there are no data on safe human use, we in parallel, can already at this early stage assess potential toxicity of the same sample. Observed activities of natural compound mixtures can be compared to compounds with known health effects or toxicity profiles, thereby assessing the uniqueness of the activity profile.

## Identification of bioactive compounds in mixtures using Effect-Directed Analysis

If a desired activity profile is obtained, indicating the presence of bioactive compounds with a positive health effect, as a next step the compound(s) responsible for this bioactivity can be purified through a step-wise fractionation coupled to bioactivity testing of the obtained active mixtures. This effect-directed analysis is continued until a relatively pure biologically active fraction is obtained that can be subjected to chemical analytics to identify the compound(s) of interest. If successful, the CALUX<sup>®</sup> methods already provide important information on its mode-of-action, while follow-up studies using suitable cell types can complement this knowledge. The identification of the compound, together with the assessment of its mode of action, and if possible combined with historic use of the crude sample can then form the basis of a health claim.



## Innovations



BDS is a front-end player in innovative bio-analytics. Our laboratories include fully equipped Innovation and Development departments with ample experience in developing new bioanalytical tools (including all current CALUX® assays) and their various applications. As such, our proprietary CALUX® methods have been transformed from the classical manual format to high throughput formats using robotics. This also allowed miniaturization to 384-wells format, increasing both throughput and accuracy. Furthermore, in this miniaturised set-up, only a fraction of the original sample volume is required, which is especially relevant when only small sample volumes are available, such as in clinical or epidemiological studies. Comprehensive panels of bioassays have been developed for safety- and activity profiling for various applications. Current research focuses on further expansion of the range of bioanalytical tools and their applications. Those include animal alternatives for activity screens and safety assessments, barcoding methods, and tool development for rapid data interpretation and extrapolation, including assessment of metabolism and pharmacokinetics. In addition, cost reduction, simplification, and on-line technologies are current focal areas of future innovations. Through the metagenomics expertise of partner SME MLS, and fractionation and chemical identification technologies, the bioactivity screening capabilities of BDS will be increasingly used for new bio-based product development. This ambitious program is carried out in close collaboration with other companies and academic groups in the context of many public private partnerships in major national- and international projects.

## Expertise

BDS employs highly trained personnel with a strong academic and commercial background. In addition, the BDS team does not operate in isolation but in an extensive network of international collaborations. This allows handling of sensitive and complex analytical problems, interpretation of the data as required in our contract research and consultancy activities. We offer our technologies and services world-wide through a network of laboratories and agents, and we thereby have access to a wide range of service analyses to complement our bioanalytical package. Through our innovative laboratories, access to the latest developments in DNA and RNA sequencing, quantitative PCR, molecular cloning, analytical chemistry, and cell culture are available to our customers. Our skilled personnel in the Service and Innovation departments can offer tailor-made contract research solutions. Our partner SME MLS provides advanced metagenomics technologies.

## Facilities

Our staff and facilities comprise knowledge, experience, and equipment for performing projects related to effect-based testing and the development and validation of new experimental tools and approaches. We use this infrastructure for improving and expanding our own portfolio, but we also offer contract analysis services and contract research. BDS is located at the Amsterdam Science Park (ASP) in the Netherlands. The Amsterdam Science Park hosts one of the largest concentrations of beta sciences in Europe. Among the residents of the ASP are the University of Amsterdam Faculty of Science, Amsterdam University College, other research institutes and some 120 companies – from

start-ups to multinationals – all working in the fields of IT, Life Sciences, advanced technology, and sustainability. We are operating internationally and can offer contract-service analyses, contract research and licencing of our CALUX® biotechnology. BDS has a state-of-the-art and fully equipped laboratory with a wide range of methods, high level of knowledge and expertise.

Our facilities are equipped for a.o.:

- ✓ Cell culturing
- ✓ Extraction of a wide variety of matrices
- ✓ Post- extraction clean-up
- ✓ Analytical chemistry
- ✓ State-of-the-art molecular biology
- ✓ Gene expression, next generation DNA and RNA sequencing
- ✓ Cell-based quantitative high-throughput screening

## R&D Projects

Being a knowledge intensive company, BDS follows the philosophy that participation in national and international research projects creates challenging and important opportunities for knowledge exchange with universities, institutions, and other companies. Moreover, we believe that those cooperations contribute to further development of our portfolio and optimization and demonstration of our technologies to different stakeholders. In addition to active participation in these projects, our scientific staff often plays a coordinating role.



## BDS' bioassay overview

Name	Pathway	agonistic module	antagonistic module	metabolic modules
DR CALUX	dioxin receptor activation	●	●	●
DRhp CALUX	dioxin receptor activation	●	●	●
DRhuman CALUX	dioxin receptor activation	●	●	●
PAH CALUX	dioxin receptor activation	●	●	●
ER CALUX	estrogen receptor activation	●	●	●
ERalpha CALUX	estrogen receptor $\alpha$ activation	●	●	●
ERbeta CALUX	estrogen receptor $\beta$ activation	●	●	●
AR CALUX	androgen receptor activation	●	●	●
PR CALUX	progesterone receptor activation	●	●	●
GR CALUX	glucocorticoid receptor activation	●	●	●
TRbeta CALUX	thyroid receptor beta activation	●	●	●
RAR CALUX	retinoic acid receptor activation	●	●	●
PPARalpha CALUX	PPAR $\gamma$ activation	●	●	●
PPARdelta CALUX	PPAR $\alpha$ activation	●	●	●
PPARGamma CALUX	PPAR $\delta$ activation	●	●	●
PXR CALUX	PXR activation	●	●	●
LXR CALUX	LXR activation	●	●	●
NFkappaB CALUX	NF $\kappa$ B pathway activation	●	●	●
P21 CALUX	transcription of p21 inhibitor of cell cycle	●	●	●
Nrf2 CALUX	activation of the Nrf2 pathway	●	●	●
P53 CALUX	genotoxicity; p53-dependent pathway activation	●	●	●
p53liver CALUX	genotoxicity; p53-dependent pathway activation	●	●	●
TCF CALUX	wnt/TCF pathway activation	●	●	●
AP1 CALUX	AP1 pathway activation	●	●	●
HIF1alpha CALUX	chemical hypoxia response	●	●	●
ER stress CALUX	endoplasmic reticulum stress response	●	●	●
cytotox CALUX	modulation constitutive transcription	●	●	●
PFAS CALUX	PFAS assay, TTR binding-based	●		●
TTR assay	binding to thyroid transporter	●		●
H295R assay	modulation steroid synthesis	●	●	●
3T3-L1 assay	modulation fat cell differentiation	●	●	

*please enquire for licensing possibilities of the CALUX® lines*

# CALUX<sup>®</sup> reporter cell line descriptions



## DR CALUX®

The dioxin responsive (DR) CALUX consists of the rat hepatoma cell line H4IIE, incorporating the firefly luciferase gene coupled to dioxin responsive elements (DREs) as a reporter gene for the presence of dioxins (PCDDs) and dioxin-like compounds, like furans (PCDFs) and dioxin-like PCBs (dl-PCBs). Following binding of these compounds to the intracellular aryl-hydrocarbon receptor (AhR), the ligand-receptor complex binds the DRE. This will lead to expression of proteins that are under normal circumstances associated to DRE-mediated transcription, but also luciferase. After addition of the appropriate substrate for luciferase, light is emitted. The amount of light produced is proportional to the amount of ligand-specific receptor activation, which is benchmarked against the relevant reference compound 2,3,7,8-TCDD, and expressed as toxic equivalents (TEQs), or bioanalytical equivalents (BEQs).

Specification	DR CALUX
Basal cell line	H4IIE
Species	rat
Tissue	liver
Positive control	2,3,7,8-TCDD
Endpoint (pure compounds)	EC or PC concentration, lowest effect concentration (e.g. PC10)
Endpoint (mixtures)	Toxic equivalents in pg TEQ/g sample processed
Test duration	24hr (incubation time)
Specificity	Binding to the AhR, through selective sulfuric acid work-up method typically by very stable dioxin-like compounds only. Ligand selections can be made through compound class selective workup methods and/or metabolic modules.
Assay interferences	Minimal because of use of highly pathway specific constructs, and extensive QA/QC. In addition, for dioxin TEQ assessment in mixtures the sample is cleaned up by a sulfuric acid treatment and afterwards with an additional step to separate dl-PCBs from PCDD/Fs. Cytotoxicity is checked to exclude false-negatives. In case of false positive/false negative guided levels must be established to compare it with (e.g., EC/644/2017 below 5% false compliant rate accepted).
Sensitivity (LOD/Q)	Typically in the pg range (matrix- and sample size-dependent)
Matrices	Any type of sample
Sample volume/mass	Matrix- and desired limit of quantification (LOQ)-dependent
Amount of compound	Typically 10 mg. Much lower for high potency compound provided in DMSO
Assessment criteria	In house methods, compliant with relevant application/regulations.
SOPs and Guidelines	BDS internal and EC COMMISSION REGULATION (EU) No 644/2017, COMMISSION REGULATION (EU) No 771/2017, NL-SPECIE-07 (Rijkswaterstaat, the Netherlands), EPA-4435 (USA), JIS guidelines 463 (Japan), Veileder for risikovurdering av forurenset sediment (TA-2085/2005) (Norway) and EURL-ECVAM method DB-ALM Protocol n° 197.: Automated CALUX reporter gene assay procedure.
HTS protocol	BDS; see EURL-ECVAM DB-ALM Protocol n° 197: Automated CALUX reporter gene assay procedure
Key reference	Garrison PM, Tullis K, Aarts JM, Brouwer A, Giesy JP, Denison MS (1996) Species-specific recombinant cell lines as bioassay systems for the detection of 2,3,7,8-tetrachlorodibenzo-p-dioxin-like chemicals. <i>Fundam Appl Toxicol.</i> 30:194-203.



## DRhp CALUX®

The high performance (hp) variant of the dioxin responsive (DR) CALUX consists of the rat hepatoma cell line H4IIE, incorporating the firefly luciferase gene coupled to dioxin responsive elements (DREs) as a reporter gene for the presence of dioxins (PCDDs) and dioxin-like compounds, like furans (PCDFs) and dioxin-like PCBs (dl-PCBs). Following binding of these compounds to the intracellular aryl-hydrocarbon receptor (AhR), the ligand-receptor complex binds the DRE. This will lead to expression of proteins that are under normal circumstances associated to DRE-mediated transcription, but also luciferase. After addition of the appropriate substrate for luciferase, light is emitted. The amount of light produced is proportional to the amount of ligand-specific receptor activation, which is benchmarked against the relevant reference compound 2,3,7,8-TCDD, and expressed as toxic equivalents (TEQs), or bioanalytical equivalents (BEQs). Being even more sensitive than the standard DR CALUX line, the hp line has been specifically designed to analyse samples of very small size, like in epidemiological studies.

Specification	DRhp CALUX
Basal cell line	H4IIE
Species	rat
Tissue	liver
Positive control	2,3,7,8-TCDD
Endpoint (pure compounds)	EC or PC concentration, lowest effect concentration (e.g. PC10)
Endpoint (mixtures)	Toxic equivalents in pg TEQ/g sample processed
Test duration	24hr (incubation time)
Specificity	Binding to the AhR, through selective sulfuric acid work-up method typically by very stable dioxin-like compounds only. Ligand selections can be made through compound class selective workup methods and/or metabolic modules.
Assay interferences	Minimal because of use of highly pathway specific construct, and extensive QA/QC. In addition, for dioxin TEQ assessment in mixtures the sample is cleaned up by a sulfuric acid treatment and afterwards with an additional step to separate dl-PCBs from PCDD/Fs. Cytotoxicity is checked to exclude false-negatives.
Sensitivity (LOD/Q)	Typically in the low pg range (matrix- and sample size-dependent)
Matrices	Any type of sample
Sample volume/mass	Matrix- and desired limit of quantification (LOQ)-dependent
Amount of compound	Typically 10 mg. Much lower for high potency compound provided in DMSO
Assessment criteria	In house methods, compliant with relevant application/regulations.
SOPs and Guidelines	BDS internal and EC COMMISSION REGULATION (EU) No 644/2017, COMMISSION REGULATION (EU) No 771/2017, NL-SPECIE-07 (Rijkswaterstaat, the Netherlands), EPA-4435 (USA), JIS guidelines 463 (Japan), Veileder for risikovurdering av forurenset sediment (TA-2085/2005) (Norway) and EURL-ECVAM method DB-ALM Protocol n° 197.: Automated CALUX reporter gene assay procedure.
HTS protocol	BDS; see EURL-ECVAM DB-ALM Protocol n° 197: Automated CALUX reporter gene assay procedure
Key reference	Budin C, Talia C, Besselink H, Van Vugt-Lussenburg B, Swart K, Jonker L, Middelhof I, Brouwer A, Fowler P, Van der Burg B (2021) Assessment of the effect of maternal smoking on placental and foetal hepatic AhR activity using a CALUX reporter gene assay with improved sensitivity, the DRhp CALUX. Thesis chapter, Vrije Universiteit Amsterdam.

## DRhuman CALUX®

The human cell-based dioxin responsive (DR) CALUX consists of the human hepatoma cell line HepG2, incorporating the firefly luciferase gene coupled to dioxin responsive elements (DREs) as a reporter gene for the presence of dioxins (PCDDs) and dioxin-like compounds, like furans (PCDFs) and dioxin-like PCBs (dl-PCBs). Following binding of these compounds to the intracellular aryl-hydrocarbon receptor (AhR), the ligand-receptor complex binds the DRE. This will lead to expression of proteins that are under normal circumstances associated to DRE-mediated transcription, but also luciferase. After addition of the appropriate substrate for luciferase, light is emitted. The amount of light produced is proportional to the amount of ligand-specific receptor activation, which is benchmarked against the relevant reference compound 2,3,7,8-TCDD, and expressed as toxic equivalents (TEQs), or bioanalytical equivalents (BEQs). While it is in general less sensitive than the rat cell-based lines, it can be applied in cases where species-specific differences in ligand responses could occur.

Specification	DRhuman CALUX
Basal cell line	HepG2
Species	human
Tissue	liver
Positive control	2,3,7,8-TCDD
Endpoint (pure compounds)	EC or PC concentration, lowest effect concentration (e.g. PC10)
Endpoint (mixtures)	Toxic equivalents in pg TEQ/g sample processed
Test duration	24hr (incubation time)
Specificity	Binding to the AhR, through selective sulfuric acid work-up method typically by very stable dioxin-like compounds only. Ligand selections can be made through compound class selective workup methods and/or metabolic modules.
Assay interferences	Minimal because of use of highly pathway specific construct, and extensive QA/QC. In addition, for dioxin TEQ assessment in mixtures the sample is cleaned up by a sulfuric acid treatment and afterwards with an additional step to separate dl-PCBs from PCDD/Fs. Cytotoxicity is checked to exclude false-negatives.
Sensitivity (LOD/Q)	Typically in the high pg range (matrix- and sample size-dependent)
Matrices	Any type of sample
Sample volume/mass	Matrix- and desired limit of quantification (LOQ)-dependent
Amount of compound	Typically 10 mg. Much lower for high potency compound provided in DMSO
Assessment criteria	In house methods, compliant with relevant application/regulations.
SOPs and Guidelines	BDS internal and EURL-ECVAM method DB-ALM Protocol n° 197: Automated CALUX reporter gene assay procedure.
HTS protocol	BDS; see EURL-ECVAM DB-ALM Protocol n° 197 : Automated CALUX reporter gene assay procedure
Key reference	Budin C, Talia C, Besselink H, Van Vugt-Lussenburg B, Swart K, Jonker L, Middelhof I, Brouwer A, Fowler P, Van der Burg B (2021) Assessment of the effect of maternal smoking on placental and foetal hepatic AhR activity using a CALUX reporter gene assay with improved sensitivity, the DRhp CALUX. Thesis chapter, Vrije Universiteit Amsterdam.

## PAH CALUX®

The polyhalogenated hydrocarbon (PAH)-responsive PAH CALUX consists of the rat hepatoma cell line H4IIE, incorporating the firefly luciferase gene coupled to dioxin responsive elements (DREs) as a reporter gene for the presence of carcinogenic PAHs. Following binding of these compounds to the intracellular aryl-hydrocarbon receptor (AhR), the ligand-receptor complex binds the DRE. This will lead to expression of proteins that are under normal circumstances associated to DRE-mediated transcription, but also luciferase. After addition of the appropriate substrate for luciferase, light is emitted. The amount of light produced is proportional to the amount of ligand-specific receptor activation, which is benchmarked against the relevant reference compound benzo(a)pyrene (BaP), and expressed as toxic equivalents (TEQs), or bioanalytical equivalents (BEQs). Through the use of a specific reporter gene construct, selective workup and bioanalysis protocols PAH selectivity is obtained.

Specification	PAH CALUX
Basal cell line	H4IIE
Species	rat
Tissue	liver
Positive control	benzo-a-pyrene
Endpoint (pure compounds)	EC or PC concentration, lowest effect concentration (e.g. PC10)
Endpoint (mixtures)	Toxic equivalents in pg TEQ/g sample processed
Test duration	6hr (incubation time)
Specificity	Binding to the AhR, through specific work-up method and short incubation time typically by PAHs only
Assay interferences	Minimal because of use of highly pathway specific construct, selective workup, and extensive QA/QC. Cytotoxicity is checked to exclude false-negatives.
Sensitivity (LOD/Q)	Typically in ng range (matrix- and sample size-dependent)
Matrices	Any type of sample
Sample volume/mass	Matrix- and desired limit of quantification (LOQ)-dependent
Amount of compound	Typically 10 mg. Much lower for high potency compound provided in DMSO
Assessment criteria	In house methods, compliant with relevant application/regulations.
SOPs and Guidelines	BDS internal and EURL-ECVAM method DB-ALM Protocol n° 197: Automated CALUX reporter gene assay procedure.
HTS protocol	BDS; see EURL-ECVAM DB-ALM Protocol n° 197 : Automated CALUX reporter gene assay procedure
Key reference	Pieterse B, Felzel E, Winter R, van der Burg B, Brouwer A (2013) PAH-CALUX, an optimized bioassay for carcinogenic hazard identification of polycyclic aromatic hydrocarbons (PAHs) as individual compounds and in complex mixtures. Environ Sci Technol, 47, 11651-11659.



## ER CALUX®

The estrogen responsive (ER) CALUX consists of the human breast cancer cell line T47D, incorporating the firefly luciferase gene coupled to estrogen responsive elements (EREs) as a reporter gene for the presence of estrogens and estrogen-like compounds. Following binding of these compounds to the intracellular estrogen receptors (alpha and beta), the ligand-receptor complex binds the ERE. This will lead to expression of proteins that are under normal circumstances associated to ERE-mediated transcription, but also luciferase. After addition of the appropriate substrate for luciferase, light is emitted. The amount of light produced is proportional to the amount of ligand-specific receptor activation, which is benchmarked against the relevant reference compound 17 $\beta$ -estradiol (E2), and expressed as toxic equivalents (TEQs), or bioanalytical equivalents (BEQs).

Specification	ER CALUX
Basal cell line	T47D
Species	human
Tissue	breast
Positive control	17 $\beta$ -estradiol
Endpoint (pure compounds)	EC or PC concentration, lowest effect concentration (e.g. PC10)
Endpoint (mixtures)	Toxic equivalents in pg TEQ/g sample processed
Test duration	24hr (incubation time)
Specificity	Binding to the endogenous estrogen receptors. Ligand selections can be made through compound class selective workup methods and/or metabolic modules.
Assay interferences	Minimal because of use of highly pathway specific construct, and extensive QA/QC. Due to the use of endogenously expressed receptors cross-talk may occur. Cytotoxicity and non-specific luciferase interferences experienced with certain ligands and samples can be assessed with the cytotox CALUX assay.
Sensitivity (LOD/Q)	Typically in the low pg range (matrix- and sample size-dependent)
Matrices	Any type of sample
Sample volume/mass	Matrix- and desired limit of quantification (LOQ)-dependent
Amount of compound	Typically 10 mg. Much lower for high potency compound provided in DMSO
Assessment criteria	In house methods, compliant with relevant application/regulations.
SOPs and Guidelines	BDS internal, Dutch Rijkswaterstaat RIKZ-Specie-08 guideline; ISO 19040-3.
HTS protocol	Not available
Key reference	Legler, J., Van den Brink, C.E., Brouwer, A., Murk, A.J., Van der Saag, P.T., Vethaak, A.D., and Van der Burg, B. (1999) Development of a stably transfected estrogen receptor-mediated luciferase reporter gene assay in the human T47-D breast cancer cell line. Toxicological Sciences 48, 55-66.

## ERalpha CALUX®

The estrogen receptor alpha responsive (ERalpha) CALUX consists of the human osteosarcoma cell line U2OS, incorporating the firefly luciferase gene coupled to estrogen responsive elements (EREs) as a reporter gene for the presence of estrogens and estrogen-like compounds, as well as human ERalpha. Following binding of these compounds to the intracellular ERalpha, the ligand-receptor complex binds the ERE. This will lead to expression of proteins that are under normal circumstances associated to ERE-mediated transcription, but also luciferase. After addition of the appropriate substrate for luciferase, light is emitted. The amount of light produced is proportional to the amount of ligand-specific receptor activation, which is benchmarked against the relevant reference compound 17 $\beta$ -estradiol (E2), and expressed as toxic equivalents (TEQs), or bioanalytical equivalents (BEQs).

Specification	ERalpha CALUX
Basal cell line	U2OS
Species	human
Tissue	bone
Positive control	17 $\beta$ -estradiol
Endpoint (pure compounds)	EC or PC concentration, lowest effect concentration (e.g. PC10)
Endpoint (mixtures)	Toxic equivalents in pg TEQ/g sample processed
Test duration	24hr (incubation time)
Specificity	Binding to ERalpha only. Ligand selections can be made through compound class selective workup methods and/or metabolic modules.
Assay interferences	Minimal because of use of highly pathway specific construct and specific receptor construct, and extensive QA/QC. Cytotoxicity and non-specific luciferase interferences experienced with certain ligands and samples can be assessed with the cytotox CALUX assay.
Sensitivity (LOD/Q)	Typically in the low pg range (matrix- and sample size-dependent)
Matrices	Any type of sample
Sample volume/mass	Matrix- and desired limit of quantification (LOQ)-dependent
Amount of compound	Typically 10 mg. Much lower for high potency compound provided in DMSO
Assessment criteria	In house methods, compliant with relevant application/regulations.
SOPs and Guidelines	BDS internal and ISO 19040-3 (water/waste water); OECD TG455; Dutch Rijkswaterstaat RIKZ-Specie-08 guideline; Australian Water Commission; EPA California.
HTS protocol	BDS; see EURL-ECVAM DB-ALM Protocol n° 197 : Automated CALUX reporter gene assay procedure
Key reference	Sonneveld, E., Jansen, H.J., Riteco, J.A.C., Brouwer, A., Van der Burg, B. (2005) Development of androgen- and estrogen-responsive bioassays, members of a panel of human cell line-based highly selective steroid responsive bioassays. Toxicol. Sci., 83, 136-48.

## ERbeta CALUX®

The estrogen receptor beta responsive (ERbeta) CALUX consists of the human osteosarcoma cell line U2OS, incorporating the firefly luciferase gene coupled to estrogen responsive elements (EREs) as a reporter gene for the presence of estrogens and estrogen-like compounds, as well as human ERbeta. Following binding of these compounds to the intracellular ERbeta, the ligand-receptor complex binds the ERE. This will lead to expression of proteins that are under normal circumstances associated to ERE-mediated transcription, but also luciferase. After addition of the appropriate substrate for luciferase, light is emitted. The amount of light produced is proportional to the amount of ligand-specific receptor activation, which is benchmarked against the relevant reference compound 17 $\beta$ -estradiol (E2), and expressed as toxic equivalents (TEQs), or bioanalytical equivalents (BEQs).

Specification	ERbeta CALUX
Basal cell line	U2OS
Species	human
Tissue	bone
Positive control	17 $\beta$ -estradiol
Endpoint (pure compounds)	EC or PC concentration, lowest effect concentration (e.g. PC10)
Endpoint (mixtures)	Toxic equivalents in pg TEQ/g sample processed
Test duration	24hr (incubation time)
Specificity	Binding to ERbeta only. Ligand selections can be made through compound class selective workup methods and/or metabolic modules.
Assay interferences	Minimal because of use of highly pathway specific construct and specific receptor construct, and extensive QA/QC. Cytotoxicity and non-specific luciferase interferences experienced with certain ligands and samples can be assessed with the cytotox CALUX assay.
Sensitivity (LOD/Q)	Typically in pg range (matrix- and sample size-dependent)
Matrices	Any type of sample
Sample volume/mass	Matrix- and desired limit of quantification (LOQ)-dependent
Amount of compound	Typically 10 mg. Much lower for high potency compound provided in DMSO
Assessment criteria	In house methods, compliant with relevant application/regulations.
SOPs and Guidelines	Similar to ERalpha, which is the preferred assay in guideline studies.
HTS protocol	BDS; see EURL-ECVAM DB-ALM Protocol n° 197 : Automated CALUX reporter gene assay procedure
Key reference	Van der Burg, B., Van der Linden, S.C., Man, H.Y., Winter, R., Jonker, L., Van Vugt-Lussenburg, B., Brouwer, A. (2013) A panel of quantitative CALUX® reporter gene assays for reliable high throughput toxicity screening of chemicals and complex mixtures. In "High throughput screening methods in toxicity testing" (P. Steinberg, ed). John Wiley and Sons, Inc. New York. ISBN 9781118065631 pp. 519-532

## AR CALUX®

The androgen receptor responsive (AR) CALUX consists of the human osteosarcoma cell line U2OS, incorporating the firefly luciferase gene coupled to androgen responsive elements (AREs) as a reporter gene for the presence of androgens and androgen-like compounds, as well as human androgen receptor. Following binding of these compounds to the intracellular AR, the ligand-receptor complex binds the AREs. This will lead to expression of proteins that are under normal circumstances associated to ARE-mediated transcription, but also luciferase. After addition of the appropriate substrate for luciferase, light is emitted. The amount of light produced is proportional to the amount of ligand-specific receptor activation, which is benchmarked against the relevant reference compound, dihydrotestosterone (DHT), and expressed as toxic equivalents (TEQs), or bioanalytical equivalents (BEQs).

Specification	AR CALUX
Basal cell line	U2OS
Species	human
Tissue	bone
Positive control	dihydrotestosterone
Endpoint (pure compounds)	EC or PC concentration, lowest effect concentration (e.g. PC10)
Endpoint (mixtures)	Toxic equivalents in pg TEQ/g sample processed
Test duration	24hr (incubation time)
Specificity	Binding to AR only. Ligand selections can be made through compound class selective workup methods and/or metabolic modules.
Assay interferences	Minimal because of use of highly pathway specific construct and specific receptor construct, and extensive QA/QC. Cytotoxicity and non-specific luciferase interferences experienced with certain ligands and samples can be assessed with the cytotox CALUX assay.
Sensitivity (LOD/Q)	Typically in pg range (matrix- and sample size-dependent)
Matrices	Any type of sample
Sample volume/mass	Matrix- and desired limit of quantification (LOQ)-dependent
Amount of compound	Typically 10 mg. Much lower for high potency compound provided in DMSO
Assessment criteria	In house methods, compliant with relevant application/regulations.
SOPs and Guidelines	BDS internal, OECD TG458, and relevant protocols similar to ER guidelines
HTS protocol	BDS; see EURL-ECVAM DB-ALM Protocol n° 197 : Automated CALUX reporter gene assay procedure
Key reference	Sonneveld, E., Jansen, H.J., Riteco, J.A.C., Brouwer, A., Van der Burg, B. (2005) Development of androgen- and estrogen-responsive bioassays, members of a panel of human cell line-based highly selective steroid responsive bioassays. Toxicol. Sci., 83, 136-48.

## PR CALUX®

The progesterone receptor responsive (PR) CALUX consists of the human osteosarcoma cell line U2OS, incorporating the firefly luciferase gene coupled to progesterone responsive elements (PREs) as a reporter gene for the presence of progestins and progestin-like compounds, as well as human progesterone receptor. Following binding of these compounds to the intracellular PR, the ligand-receptor complex binds the PREs. This will lead to expression of proteins that are under normal circumstances associated to PRE-mediated transcription, but also luciferase. After addition of the appropriate substrate for luciferase, light is emitted. The amount of light produced is proportional to the amount of ligand-specific receptor activation, which is benchmarked against the relevant reference compound, progesterone, and expressed as toxic equivalents (TEQs), or bioanalytical equivalents (BEQs).

Specification	PR CALUX
Basal cell line	U2OS
Species	human
Tissue	bone
Positive control	progesterone
Endpoint (pure compounds)	EC or PC concentration, lowest effect concentration (e.g. PC10)
Endpoint (mixtures)	Toxic equivalents in pg TEQ/g sample processed
Test duration	24hr (incubation time)
Specificity	Binding to PR only. Ligand selections can be made through compound class selective workup methods and/or metabolic modules.
Assay interferences	Minimal because of use of highly pathway specific construct and specific receptor construct, and extensive QA/QC. Cytotoxicity and non-specific luciferase interferences experienced with certain ligands and samples can be assessed with the cytotox CALUX assay.
Sensitivity (LOD/Q)	Typically in pg range (matrix- and sample size-dependent)
Matrices	Any type of sample
Sample volume/mass	Matrix- and desired limit of quantification (LOQ)-dependent
Amount of compound	Typically 10 mg. Much lower for high potency compound provided in DMSO
Assessment criteria	In house methods, compliant with relevant application/regulations.
SOPs and Guidelines	BDS internal, similar to ER-, and AR CALUX assays
HTS protocol	BDS; see EURL-ECVAM DB-ALM Protocol n° 197 : Automated CALUX reporter gene assay procedure
Key reference	Sonneveld, E., Jansen, H.J., Riteco, J.A.C., Brouwer, A., Van der Burg, B. (2005) Development of androgen- and estrogen-responsive bioassays, members of a panel of human cell line-based highly selective steroid responsive bioassays. Toxicol. Sci., 83, 136-48.



## GR CALUX®

The glucocorticoid receptor responsive (GR) CALUX consists of the human osteosarcoma cell line U2OS, incorporating the firefly luciferase gene coupled to glucocorticoid responsive elements (PREs) as a reporter gene for the presence of glucocorticoids and glucocorticoid-like compounds, as well as human glucocorticoid receptor. Following binding of these compounds to the intracellular GR, the ligand-receptor complex binds the GREs. This will lead to expression of proteins that are under normal circumstances associated to GRE-mediated transcription, but also luciferase. After addition of the appropriate substrate for luciferase, light is emitted. The amount of light produced is proportional to the amount of ligand-specific receptor activation, which is benchmarked against the relevant reference compound, dexamethasone (Dex), and expressed as toxic equivalents (TEQs), or bioanalytical equivalents (BEQs).

Specification	GR CALUX
Basal cell line	U2OS
Species	human
Tissue	bone
Positive control	dexamethasone
Endpoint (pure compounds)	EC or PC concentration, lowest effect concentration (e.g. PC10)
Endpoint (mixtures)	Toxic equivalents in pg TEQ/g sample processed
Test duration	24hr (incubation time)
Specificity	Binding to GR only. Ligand selections can be made through compound class selective workup methods and/or metabolic modules.
Assay interferences	Minimal because of use of highly pathway specific construct and specific receptor construct, and extensive QA/QC. Cytotoxicity and non-specific luciferase interferences experienced with certain ligands and samples can be assessed with the cytotox CALUX assay.
Sensitivity (LOD/Q)	Typically in high pg range (matrix- and sample size-dependent)
Matrices	Any type of sample
Sample volume/mass	Matrix- and desired limit of quantification (LOQ)-dependent
Amount of compound	Typically 10 mg. Much lower for high potency compound provided in DMSO
Assessment criteria	In house methods, compliant with relevant application/regulations.
SOPs and Guidelines	BDS internal, similar to ER-, and AR CALUX assays
HTS protocol	BDS; see EURL-ECVAM DB-ALM Protocol n° 197 : Automated CALUX reporter gene assay procedure
Key reference	Sonneveld, E., Jansen, H.J., Riteco, J.A.C., Brouwer, A., Van der Burg, B. (2005) Development of androgen- and estrogen-responsive bioassays, members of a panel of human cell line-based highly selective steroid responsive bioassays. Toxicol. Sci., 83, 136-48.

## TRbeta CALUX®

The thyroid receptor beta responsive (TRbeta) CALUX consists of the human osteosarcoma cell line U2OS, incorporating the firefly luciferase gene coupled to thyroid responsive elements (TREs) as a reporter gene for the presence of thyroid hormones and thyroid hormone-like compounds, as well as human thyroid receptor beta. Following binding of these compounds to the intracellular TRbeta, the ligand-receptor complex binds the TREs. This will lead to expression of proteins that are under normal circumstances associated to TRE-mediated transcription, but also luciferase. After addition of the appropriate substrate for luciferase, light is emitted. The amount of light produced is proportional to the amount of ligand-specific receptor activation, which is benchmarked against the relevant reference compound, triiodothyronine (T3), and expressed as toxic equivalents (TEQs), or bioanalytical equivalents (BEQs).

Specification	TRbeta CALUX
Basal cell line	U2OS
Species	human
Tissue	bone
Positive control	triiodothyronine
Endpoint (pure compounds)	EC or PC concentration, lowest effect concentration (e.g. PC10)
Endpoint (mixtures)	Toxic equivalents in pg TEQ/g sample processed
Test duration	24hr (incubation time)
Specificity	Binding to TR. Ligand selections can be made through compound class selective workup methods and/or metabolic modules.
Assay interferences	Minimal because of use of highly pathway specific construct and specific receptor construct, and extensive QA/QC. Cytotoxicity and non-specific luciferase interferences experienced with certain ligands and samples can be assessed with the cytotox CALUX assay.
Sensitivity (LOD/Q)	Typically in high pg range (matrix- and sample size-dependent)
Matrices	Any type of sample
Sample volume/mass	Matrix- and desired limit of quantification (LOQ)-dependent
Amount of compound	Typically 10 mg. Much lower for high potency compound provided in DMSO
Assessment criteria	In house methods, compliant with relevant application/regulations.
SOPs and Guidelines	BDS internal, similar to ER-, and AR CALUX assays
HTS protocol	BDS; see EURL-ECVAM DB-ALM Protocol n° 197 : Automated CALUX reporter gene assay procedure
Key reference	Collet B, Simon E, Van der Linden S, El Abdellaoui N, Naderman M, Man HY, Middelhof I, Van der Burg B, Besselink H, Brouwer A. (2019) Validation of a panel of in vitro methods for assessing thyroid receptor $\beta$ and transthyretin transporter disrupting activities. Reproductive Toxicol., 18, 30554-30559

## RAR CALUX®

The retinoic acid responsive responsive (RAR) CALUX consists of the human osteosarcoma cell line U2OS, incorporating the firefly luciferase gene coupled to retinoic acid responsive elements (RAREs) as a reporter gene for the presence of retinoids and retinoid-like compounds. Following binding of these compounds to the intracellular retinoic acid receptor, the ligand-receptor complex binds the RAREs. This will lead to expression of proteins that are under normal circumstances associated to RARE-mediated transcription, but also luciferase. After addition of the appropriate substrate for luciferase, light is emitted. The amount of light produced is proportional to the amount of ligand-specific receptor activation, which is benchmarked against the relevant reference compound, retinoic acid (RA), and expressed as toxic equivalents (TEQs), or bioanalytical equivalents (BEQs).

Specification	RAR CALUX
Basal cell line	U2OS
Species	human
Tissue	bone
Positive control	retinoic acid
Endpoint (pure compounds)	EC or PC concentration, lowest effect concentration (e.g. PC10)
Endpoint (mixtures)	Toxic equivalents in pg TEQ/g sample processed
Test duration	24hr (incubation time)
Specificity	Binding to RAR. Ligand selections can be made through compound class selective workup methods and/or metabolic modules.
Assay interferences	Minimal because of use of highly pathway specific construct and specific receptor construct, and extensive QA/QC. Cytotoxicity and non-specific luciferase interferences experienced with certain ligands and samples can be assessed with the cytotox CALUX assay.
Sensitivity (LOD/Q)	Typically in high pg range (matrix- and sample size-dependent)
Matrices	Any type of sample
Sample volume/mass	Matrix- and desired limit of quantification (LOQ)-dependent
Amount of compound	Typically 10 mg. Much lower for high potency compound provided in DMSO
Assessment criteria	In house methods, compliant with relevant application/regulations.
SOPs and Guidelines	BDS internal, similar to ER-, and AR CALUX assays
HTS protocol	BDS; see EURL-ECVAM DB-ALM Protocol n° 197 : Automated CALUX reporter gene assay procedure
Key reference	Piersma AH, Schulpen SHW, Uibel F, Van Vugt-Lussenburg, B, Bosgra S, Hermesen SAB, Roelofs MJE, Man, H., Jonker, L., Van der Linden, S, Van Duursen MBM, Wolterbeek APM, , Schwarz M, Kroese ED, Van der Burg B. (2013) Evaluation of an alternative in vitro test battery for detecting reproductive toxicants. Reprod. Toxicol. 38,53-64.



## PPARalpha CALUX®

The peroxisome proliferator receptor alpha responsive (PPARalpha) CALUX consists of the human osteosarcoma cell line U2OS, incorporating the human PPARalpha and the firefly luciferase gene coupled to PPAR responsive elements (PPREs) as a reporter gene for the presence of PPAR ligands. Following binding of these compounds to the intracellular PPARalpha, the ligand-receptor complex binds the PPREs. This will lead to expression of proteins that are under normal circumstances associated to PPRE-mediated transcription, but also luciferase. After addition of the appropriate substrate for luciferase, light is emitted. The amount of light produced is proportional to the amount of ligand-specific receptor activation, which is benchmarked against the relevant reference compound GW7674, and expressed as toxic equivalents (TEQs), or bioanalytical equivalents (BEQs).

Specification	PPARalpha CALUX
Basal cell line	U2OS
Species	human
Tissue	bone
Positive control	GW7674
Endpoint (pure compounds)	EC or PC concentration, lowest effect concentration (e.g. PC10)
Endpoint (mixtures)	Toxic equivalents in pg TEQ/g sample processed
Test duration	24hr (incubation time)
Specificity	Binding to PPARalpha. Ligand selections can be made through compound class selective workup methods and/or metabolic modules.
Assay interferences	Minimal because of use of highly pathway specific construct and specific receptor construct, and extensive QA/QC. Cytotoxicity and non-specific luciferase interferences experienced with certain ligands and samples can be assessed with the cytotox CALUX assay.
Sensitivity (LOD/Q)	Typically in high pg range (matrix- and sample size-dependent)
Matrices	Any type of sample
Sample volume/mass	Matrix- and desired limit of quantification (LOQ)-dependent
Amount of compound	Typically 10 mg. Much lower for high potency compound provided in DMSO
Assessment criteria	In house methods, compliant with relevant application/regulations.
SOPs and Guidelines	BDS internal, similar to ER-, and AR CALUX assays
HTS protocol	BDS; see EURL-ECVAM DB-ALM Protocol n° 197 : Automated CALUX reporter gene assay procedure
Key reference	Pham Ngoc L, Man HY, Besselink H, Dang Thi Cam H, Abraham Brouwer A, and Van der Burg, B. (2019) Identification of PPAR-activating compounds in herbal and edible plants and fungi from Vietnam using a comprehensive panel of PPAR reporter gene assays. Industrial Crops and Products 129, 195-200.

## PPARdelta CALUX®

The peroxisome proliferator receptor delta responsive (PPARdelta) CALUX consists of the human osteosarcoma cell line U2OS, incorporating the human PPARdelta and the firefly luciferase gene coupled to PPAR responsive elements (PPREs) as a reporter gene for the presence of PPAR ligands. Following binding of these compounds to the intracellular PPARdelta, the ligand-receptor complex binds the PPREs. This will lead to expression of proteins that are under normal circumstances associated to PPRE-mediated transcription, but also luciferase. After addition of the appropriate substrate for luciferase, light is emitted. The amount of light produced is proportional to the amount of ligand-specific receptor activation, which is benchmarked against the relevant reference compound L165041, and expressed as toxic equivalents (TEQs), or bioanalytical equivalents (BEQs).

Specification	PPARdelta CALUX
Basal cell line	U2OS
Species	human
Tissue	bone
Positive control	L165041
Endpoint (pure compounds)	EC or PC concentration, lowest effect concentration (e.g. PC10)
Endpoint (mixtures)	Toxic equivalents in pg TEQ/g sample processed
Test duration	24hr (incubation time)
Specificity	Binding to PPARdelta. Ligand selections can be made through compound class selective workup methods and/or metabolic modules
Assay interferences	Minimal because of use of highly pathway specific construct and specific receptor construct, and extensive QA/QC. Cytotoxicity and non-specific luciferase interferences experienced with certain ligands and samples can be assessed with the cytotox CALUX assay.
Sensitivity (LOD/Q)	Typically in high pg range (matrix- and sample size-dependent)
Matrices	Any type of sample
Sample volume/mass	Matrix- and desired limit of quantification (LOQ)-dependent
Amount of compound	Typically 10 mg. Much lower for high potency compound provided in DMSO
Assessment criteria	In house methods, compliant with relevant application/regulations.
SOPs and Guidelines	BDS internal, similar to ER-, and AR CALUX assays
HTS protocol	BDS; see EURL-ECVAM DB-ALM Protocol n° 197 : Automated CALUX reporter gene assay procedure
Key reference	Pham Ngoc L, Man HY, Besselink H, Dang Thi Cam H, Abraham Brouwer A, and Van der Burg, B. (2019) Identification of PPAR-activating compounds in herbal and edible plants and fungi from Vietnam using a comprehensive panel of PPAR reporter gene assays. Industrial Crops and Products 129, 195-200.

## PPARgamma CALUX®

The peroxisome proliferator receptor gamma responsive (PPARgamma) CALUX consists of the human osteosarcoma cell line U2OS, incorporating the human PPARgamma and the firefly luciferase gene coupled to PPAR responsive elements (PPREs) as a reporter gene for the presence of PPAR ligands. Following binding of these compounds to the intracellular PPARgamma, the ligand-receptor complex binds the PPREs. This will lead to expression of proteins that are under normal circumstances associated to PPRE-mediated transcription, but also luciferase. After addition of the appropriate substrate for luciferase, light is emitted. The amount of light produced is proportional to the amount of ligand-specific receptor activation, which is benchmarked against the relevant reference compound rosiglitazone and expressed as toxic equivalents (TEQs), or bioanalytical equivalents (BEQs).

Specification	PPARgamma CALUX
Basal cell line	U2OS
Species	human
Tissue	bone
Positive control	Rosiglitazone
Endpoint (pure compounds)	EC or PC concentration, lowest effect concentration (e.g. PC10)
Endpoint (mixtures)	Toxic equivalents in pg TEQ/g sample processed
Test duration	24hr (incubation time)
Specificity	Binding to PPARgamma. Ligand selections can be made through compound class selective workup methods and/or metabolic modules.
Assay interferences	Minimal because of use of highly pathway specific construct and specific receptor construct, and extensive QA/QC. Cytotoxicity and non-specific luciferase interferences experienced with certain ligands and samples can be assessed with the cytotox CALUX assay.
Sensitivity (LOD/Q)	Typically in high pg range (matrix- and sample size-dependent)
Matrices	Any type of sample
Sample volume/mass	Matrix- and desired limit of quantification (LOQ)-dependent
Amount of compound	Typically 10 mg. Much lower for high potency compound provided in DMSO
Assessment criteria	In house methods, compliant with relevant application/regulations.
SOPs and Guidelines	BDS internal, similar to ER-, and AR CALUX assays
HTS protocol	BDS; see EURL-ECVAM DB-ALM Protocol n° 197 : Automated CALUX reporter gene assay procedure
Key reference	Gijsbers, L., Man, H.Y., Kloet, S.K., De Haan, L.H.J., Keijer, J., Rietjens, I.M., Van der Burg, B., Aarts, J.M. (2011) Stable reporter cell lines for PPARγ-mediated modulation of gene expression. Analytical Biochemistry 414,77-83.

## PXR CALUX®

The pregnane X receptor responsive (PXR) CALUX consists of the human osteosarcoma cell line U2OS, incorporating the human PXR and the firefly luciferase gene coupled to a PXR responsive reporter gene for the presence of PXR ligands. Following binding of these compounds to the intracellular PXR, the ligand-receptor complex binds the reporter gene. This will lead to expression of proteins that are under normal circumstances associated to PXR-mediated transcription, but also luciferase. After addition of the appropriate substrate for luciferase, light is emitted. The amount of light produced is proportional to the amount of ligand-specific receptor activation, which is benchmarked against the relevant reference compound nicardipine, and expressed as toxic equivalents (TEQs), or bioanalytical equivalents (BEQs).

Specification	PXR CALUX
Basal cell line	U2OS
Species	human
Tissue	bone
Positive control	nicardipine
Endpoint (pure compounds)	EC or PC concentration, lowest effect concentration (e.g. PC10)
Endpoint (mixtures)	Toxic equivalents in pg TEQ/g sample processed
Test duration	24hr (incubation time)
Specificity	Binding to PXR only. Ligand selections can be made through compound class selective workup methods and/or metabolic modules.
Assay interferences	Minimal because of use of highly pathway specific construct and specific receptor construct, and extensive QA/QC. Cytotoxicity and non-specific luciferase interferences experienced with certain ligands and samples can be assessed with the cytotox CALUX assay.
Sensitivity (LOD/Q)	Typically in high pg range (matrix- and sample size-dependent)
Matrices	Any type of sample
Sample volume/mass	Matrix- and desired limit of quantification (LOQ)-dependent
Amount of compound	Typically 10 mg. Much lower for high potency compound provided in DMSO
Assessment criteria	In house methods, compliant with relevant application/regulations.
SOPs and Guidelines	BDS internal, similar to ER-, and AR CALUX assays
HTS protocol	BDS; see EURL-ECVAM DB-ALM Protocol n° 197 : Automated CALUX reporter gene assay procedure
Key reference	Piersma AH, Schulpen SHW, Uibel F, Van Vugt-Lussenburg, B, Bosgra S, Hermesen SAB, Roelofs MJE, Man, H., Jonker, L., Van der Linden, S, Van Duursen MBM, Wolterbeek APM, , Schwarz M, Kroese ED, Van der Burg B. (2013) Evaluation of an alternative in vitro test battery for detecting reproductive toxicants. Reprod. Toxicol. 38,53-64.

## LXR CALUX®

The liver X receptor responsive (LXR) CALUX consists of the human osteosarcoma cell line U2OS, incorporating the human PXR and the firefly luciferase gene coupled to PXR responsive elements (PXRREs) as a reporter gene for the presence of PXR ligands. Following binding of these compounds to the intracellular PXR, the ligand-receptor complex binds the PXRREs. This will lead to expression of proteins that are under normal circumstances associated to PXRRE-mediated transcription, but also luciferase. After addition of the appropriate substrate for luciferase, light is emitted. The amount of light produced is proportional to the amount of ligand-specific receptor activation, which is benchmarked against the relevant reference compound, the synthetic LXR agonist GW3965, and expressed as toxic equivalents (TEQs), or bioanalytical equivalents (BEQs).

Specification	LXR CALUX
Basal cell line	U2OS
Species	human
Tissue	bone
Positive control	GW3965
Endpoint (pure compounds)	EC or PC concentration, lowest effect concentration (e.g. PC10)
Endpoint (mixtures)	Toxic equivalents in pg TEQ/g sample processed
Test duration	24hr (incubation time)
Specificity	Binding to LXR only. Ligand selections can be made through compound class selective workup methods and/or metabolic modules.
Assay interferences	Minimal because of use of highly pathway specific construct and specific receptor construct, and extensive QA/QC. Cytotoxicity and non-specific luciferase interferences experienced with certain ligands and samples can be assessed with the cytotox CALUX assay.
Sensitivity (LOD/Q)	Typically in high pg range (matrix- and sample size-dependent)
Matrices	Any type of sample
Sample volume/mass	Matrix- and desired limit of quantification (LOQ)-dependent
Amount of compound	Typically 10 mg. Much lower for high potency compound provided in DMSO
Assessment criteria	In house methods, compliant with relevant application/regulations.
SOPs and Guidelines	BDS internal, similar to ER-, and AR CALUX assays
HTS protocol	BDS; see EURL-ECVAM DB-ALM Protocol n° 197 : Automated CALUX reporter gene assay procedure
Key reference	Escher S, Albrecht W, Aguayo-Orozco A, Benfenati E, Bitsch A, Braunbeck T, Brecklinghaus T, Brotzmann K, Bois F, Van der Burg B, Castel J, Exner T, Gadaleta D, Gardner I, Golbamaki N, Graepel R, Hengstler J, Jennings P, Limonciel A, Long A, MacLennan R, Mombelli E, Norinder U, Santos Capinha L, Stöber R, Taboureau O, Tolosa L, Vrijenhoek N, Van Vugt-Lussenburg B, Walker P, Van de Water B, Wehr M, White A, Zdrzil B, Fisher C. (2022) Integrate mechanistic evidence from new approach methodologies (NAMs) into a read-across assessment to characterise trends in shared mode of action. Toxicology in Vitro 79, 105269



## NFkappaB CALUX®

The NFkappaB responsive CALUX consists of the human osteosarcoma cell line U2OS, incorporating the firefly luciferase gene coupled to NFkappaB responsive elements (NFkappaB REs) as a reporter gene for the presence of NFkappaB pathway activating ligands. Following activation by these compounds of the NFkappaB pathway, NFkappaB binds to NFkappaB REs. This will lead to expression of proteins that are under normal circumstances associated to NFkappaB REs - mediated transcription, but also luciferase. After addition of the appropriate substrate for luciferase, light is emitted. The amount of light produced is proportional to the amount of ligand-specific pathway activation, which is benchmarked against the relevant reference compound, the pathway agonist interleukin-1 or 12-O-Tetradecanoylphorbol 13-acetate (TPA), and expressed as toxic equivalents (TEQs), or bioanalytical equivalents (BEQs).

Specification	NFkappaB CALUX
Basal cell line	U2OS
Species	human
Tissue	bone
Positive control	TPA or IL-1
Endpoint (pure compounds)	EC or PC concentration, lowest effect concentration (e.g. PC10)
Endpoint (mixtures)	Toxic equivalents in pg TEQ/g sample processed
Test duration	24hr (incubation time)
Specificity	Activation of the NFkappaB pathway only.. Ligand selections can be made through compound class selective workup methods and/or metabolic modules.
Assay interferences	Minimal because of use of highly pathway specific construct and specific receptor construct, and extensive QA/QC. Cytotoxicity and non-specific luciferase interferences experienced with certain ligands and samples can be assessed with the cytotox CALUX assay.
Sensitivity (LOD/Q)	Typically in high pg range (matrix- and sample size-dependent)
Matrices	Any type of sample
Sample volume/mass	Matrix- and desired limit of quantification (LOQ)-dependent
Amount of compound	Typically 10 mg. Lower for high potency compound provided in DMSO
Assessment criteria	In house methods, compliant with relevant application/regulations.
SOPs and Guidelines	BDS internal, similar to ER-, and AR CALUX assays
HTS protocol	BDS; see EURL-ECVAM DB-ALM Protocol n° 197 : Automated CALUX reporter gene assay procedure
Key reference	Piersma AH, Schulpen SHW, Uibel F, Van Vugt-Lussenburg, B, Bosgra S, Hermesen SAB, Roelofs MJE, Man, H., Jonker, L., Van der Linden, S, Van Duursen MBM, Wolterbeek APM, , Schwarz M, Kroese ED, Van der Burg B. (2013) Evaluation of an alternative in vitro test battery for detecting reproductive toxicants. <i>Reprod. Toxicol.</i> 38,53-64.



## P21 CALUX®

The p21 gene activating pathway responsive (p21) CALUX consists of the human osteosarcoma cell line U2OS, incorporating the firefly luciferase gene coupled to the promoter of the p21 gene. This gene is associated with DNA damage induced cell cycle arrest. Following activation by compounds of the p21 inducing pathway this will lead to cell cycle arrest, but also luciferase expression. After addition of the appropriate substrate for luciferase, light is emitted. The amount of light produced is proportional to the amount of ligand-specific pathway activation, which is benchmarked against the relevant reference compound, the pathway agonist actinomycin D and expressed as toxic equivalents (TEQs), or bioanalytical equivalents (BEQs).

Specification	P21 CALUX
Basal cell line	U2OS
Species	human
Tissue	bone
Positive control	actinomycin D
Endpoint (pure compounds)	EC or PC concentration, lowest effect concentration (e.g. PC10)
Endpoint (mixtures)	Toxic equivalents in pg TEQ/g sample processed
Test duration	24hr (incubation time)
Specificity	Activation of p21 promoter. Ligand selections can be made through compound class selective workup methods and/or metabolic modules.
Assay interferences	Relatively broad because of use of a p21 promoter construct. Extensive QA/QC. Cytotoxicity and non-specific luciferase interferences experienced with certain ligands and samples can be assessed with the cytotox CALUX assay.
Sensitivity (LOD/Q)	Typically in high pg range (matrix- and sample size-dependent)
Matrices	Any type of sample
Sample volume/mass	Matrix- and desired limit of quantification (LOQ)-dependent
Amount of compound	Typically 10 mg. Lower for high potency compound provided in DMSO
Assessment criteria	In house methods, compliant with relevant application/regulations.
SOPs and Guidelines	BDS internal, similar to ER-, and AR CALUX assays
HTS protocol	BDS; see EURL-ECVAM DB-ALM Protocol n° 197 : Automated CALUX reporter gene assay procedure
Key reference	Piersma AH, Schulpen SHW, Uibel F, Van Vugt-Lussenburg, B, Bosgra S, Hermesen SAB, Roelofs MJE, Man, H., Jonker, L., Van der Linden, S, Van Duursen MBM, Wolterbeek APM, , Schwarz M, Kroese ED, Van der Burg B. (2013) Evaluation of an alternative in vitro test battery for detecting reproductive toxicants. <i>Reprod. Toxicol.</i> 38,53-64.

## Nrf2 CALUX®

The Nrf2 responsive CALUX consists of the human osteosarcoma cell line U2OS, incorporating the firefly luciferase gene coupled to Nrf2 responsive elements (Nrf2 REs) as a reporter gene for the presence of Nrf2 pathway activating ligands. Following activation by these compounds of the Nrf2 pathway, Nrf2 binds to Nrf2 REs. This will lead to expression of proteins that are under normal circumstances associated to Nrf2 REs -mediated transcription, but also luciferase. After addition of the appropriate substrate for luciferase, light is emitted. The amount of light produced is proportional to the amount of ligand-specific pathway activation, which is benchmarked against the relevant reference compound, the pathway agonist curcumin, and expressed as toxic equivalents (TEQs), or bioanalytical equivalents (BEQs).

Specification	Nrf2 CALUX
Basal cell line	U2OS
Species	human
Tissue	bone
Positive control	curcumin
Endpoint (pure compounds)	EC or PC concentration, lowest effect concentration (e.g. PC10)
Endpoint (mixtures)	Toxic equivalents in pg TEQ/g sample processed
Test duration	24hr (incubation time)
Specificity	Activation of the Nrf2 pathway only.. Ligand selections can be made through compound class selective workup methods and/or metabolic modules.
Assay interferences	Minimal because of use of highly pathway specific construct, and extensive QA/QC. Cytotoxicity and non-specific luciferase interferences experienced with certain ligands and samples can be assessed with the cytotox CALUX assay.
Sensitivity (LOD/Q)	Typically in high pg range (matrix- and sample size-dependent)
Matrices	Any type of sample
Sample volume/mass	Matrix- and desired limit of quantification (LOQ)-dependent
Amount of compound	Typically 10 mg. Lower for high potency compound provided in DMSO
Assessment criteria	In house methods, compliant with relevant application/regulations.
SOPs and Guidelines	BDS internal, similar to ER-, and AR CALUX assays
HTS protocol	BDS; see EURL-ECVAM DB-ALM Protocol n° 197 : Automated CALUX reporter gene assay procedure
Key reference	Van der Linden, SC, von Bergh A, Van Vugt-Lussenburg B, Jonker L, Brouwer A, Teunis M, Krul C and Van der Burg B. (2014) Development of a panel of high throughput reporter gene assays to detect genotoxicity and oxidative stress, Mutation Res.760,23-32.

## P53 CALUX®

The p53 responsive CALUX consists of the human osteosarcoma cell line U2OS, incorporating the firefly luciferase gene coupled to p53 responsive elements (p53 REs) as a reporter gene for the presence of p53 pathway activating ligands. Following activation by these compounds of the p53 pathway, p53 binds to p53 REs. This will lead to expression of proteins that are under normal circumstances associated to p53 REs -mediated transcription, but also luciferase. After addition of the appropriate substrate for luciferase, light is emitted. The amount of light produced is proportional to the amount of ligand-specific pathway activation, which is benchmarked against the relevant reference compound, the pathway agonist actinomycin D, and expressed as toxic equivalents (TEQs), or bioanalytical equivalents (BEQs).

Specification	P53 CALUX
Basal cell line	U2OS
Species	human
Tissue	bone
Positive control	actinomycin D
Endpoint (pure compounds)	EC or PC concentration, lowest effect concentration (e.g. PC10)
Endpoint (mixtures)	Toxic equivalents in pg TEQ/g sample processed
Test duration	24hr (incubation time)
Specificity	Activation of the p53 pathway only. Ligand selections can be made through compound class selective workup methods and/or metabolic modules.
Assay interferences	Minimal because of use of highly pathway specific construct, and extensive QA/QC. Cytotoxicity and non-specific luciferase interferences experienced with certain ligands and samples can be assessed with the cytotox CALUX assay.
Sensitivity (LOD/Q)	Typically in high pg range (matrix- and sample size-dependent)
Matrices	Any type of sample
Sample volume/mass	Matrix- and desired limit of quantification (LOQ)-dependent
Amount of compound	Typically 10 mg. Lower for high potency compound provided in DMSO
Assessment criteria	In house methods, compliant with relevant application/regulations.
SOPs and Guidelines	BDS internal, similar to ER-, and AR CALUX assays
HTS protocol	BDS; see EURL-ECVAM DB-ALM Protocol n° 197 : Automated CALUX reporter gene assay procedure
Key reference	Van der Linden, SC, von Bergh A, Van Vugt-Lussenburg B, Jonker L, Brouwer A, Teunis M, Krul C and Van der Burg B. (2014) Development of a panel of high throughput reporter gene assays to detect genotoxicity and oxidative stress, Mutation Res.760,23-32.

## P53liver CALUX®

The p53 responsive CALUX consists of the human hepatoma cell line HepG2, incorporating the firefly luciferase gene coupled to p53 responsive elements (p53 REs) as a reporter gene for the presence of p53 pathway activating ligands. Following activation by these compounds of the p53 pathway, p53 binds to p53 REs. This will lead to expression of proteins that are under normal circumstances associated to p53 REs-mediated transcription, but also luciferase. After addition of the appropriate substrate for luciferase, light is emitted. The amount of light produced is proportional to the amount of ligand-specific pathway activation, which is benchmarked against the relevant reference compound, the pathway agonist actinomycin D and expressed as toxic equivalents (TEQs), or bioanalytical equivalents (BEQs).

Specification	P53liver CALUX
Basal cell line	HepG2
Species	human
Tissue	liver
Positive control	actinomycin D
Endpoint (pure compounds)	EC or PC concentration, lowest effect concentration (e.g. PC10)
Endpoint (mixtures)	Toxic equivalents in pg TEQ/g sample processed
Test duration	24hr (incubation time)
Specificity	Activation of the p53 pathway only. Ligand selections can be made through compound class selective workup methods and/or metabolic modules.
Assay interferences	Minimal because of use of highly pathway specific construct, and extensive QA/QC. Cytotoxicity and non-specific luciferase interferences experienced with certain ligands and samples can be assessed with the cytotox CALUX assay.
Sensitivity (LOD/Q)	Typically in high pg range (matrix- and sample size-dependent)
Matrices	Any type of sample
Sample volume/mass	Matrix- and desired limit of quantification (LOQ)-dependent
Amount of compound	Typically 10 mg. Lower for high potency compound provided in DMSO
Assessment criteria	In house methods, compliant with relevant application/regulations.
SOPs and Guidelines	BDS internal, similar to ER-, and AR CALUX assays
HTS protocol	BDS; see EURL-ECVAM DB-ALM Protocol n° 197 : Automated CALUX reporter gene assay procedure
Key reference	Budin C, Man HY, Al-Ayoubi C, Puel S, van Vugt-Lussenburg BMA, Brouwer A, Oswald IP, van der Burg B, Soler L. Versicolorin A enhances the genotoxicity of Aflatoxin B1 in human liver cells by inducing the transactivation of the Ah-Receptor. Food Chem Toxicol. 2021 May 10:112258.

## TCF CALUX®

The TCF responsive CALUX consists of the human osteosarcoma cell line U2OS, incorporating the firefly luciferase gene coupled to TCF responsive elements (TCF REs) as a reporter gene for the presence of TCF pathway activating ligands. Following activation by these compounds of the TCF pathway, TCF binds to TCF REs. This will lead to expression of proteins that are under normal circumstances associated to TCF REs -mediated transcription, but also luciferase. After addition of the appropriate substrate for luciferase, light is emitted. The amount of light produced is proportional to the amount of ligand-specific pathway activation, which is benchmarked against the relevant reference compound, the pathway agonists lithium chloride or wnt ligands, and expressed as toxic equivalents (TEQs), or bioanalytical equivalents (BEQs).

Specification	TCF CALUX
Basal cell line	U2OS
Species	human
Tissue	bone
Positive control	lithium chloride or wnt ligands
Endpoint (pure compounds)	EC or PC concentration, lowest effect concentration (e.g. PC10)
Endpoint (mixtures)	Toxic equivalents in pg TEQ/g sample processed
Test duration	24hr (incubation time)
Specificity	Activation of the TCF pathway only. Ligand selections can be made through compound class selective workup methods and/or metabolic modules.
Assay interferences	Minimal because of use of highly pathway specific construct, and extensive QA/QC. Cytotoxicity and non-specific luciferase interferences experienced with certain ligands and samples can be assessed with the cytotox CALUX assay.
Sensitivity (LOD/Q)	Typically in high pg range (matrix- and sample size-dependent)
Matrices	Any type of sample
Sample volume/mass	Matrix- and desired limit of quantification (LOQ)-dependent
Amount of compound	Typically 10 mg. Lower for high potency compound provided in DMSO
Assessment criteria	In house methods, compliant with relevant application/regulations.
SOPs and Guidelines	BDS internal, similar to ER-, and AR CALUX assays
HTS protocol	BDS; see EURL-ECVAM DB-ALM Protocol n° 197 : Automated CALUX reporter gene assay procedure
Key reference	Piersma AH, Schulp SHW, Uibel F, Van Vugt-Lussenburg, B, Bosgra S, Hermesen SAB, Roelofs MJE, Man, H., Jonker, L., Van der Linden, S, Van Duursen MBM, Wolterbeek APM, Schwarz M, Kroese ED, Van der Burg B. (2013) Evaluation of an alternative in vitro test battery for detecting reproductive toxicants. Reprod. Toxicol. 38,53-64.



## AP1 CALUX®

The AP1 responsive CALUX consists of the human osteosarcoma cell line U2OS, incorporating the firefly luciferase gene coupled to AP1/TPA responsive elements (TREs) as a reporter gene for the presence of AP1 pathway activating ligands. Following activation by these compounds of the AP1 pathway, AP1 binds to AP1 REs. This will lead to expression of proteins that are under normal circumstances associated to AP1 REs -mediated transcription, but also luciferase. After addition of the appropriate substrate for luciferase, light is emitted. The amount of light produced is proportional to the amount of ligand-specific pathway activation, which is benchmarked against the relevant reference compound, the pathway agonist 12-O-Tetradecanoylphorbol 13-acetate (TPA), and expressed as toxic equivalents (TEQs), or bioanalytical equivalents (BEQs).

Specification	AP1 CALUX
Basal cell line	U2OS
Species	human
Tissue	bone
Positive control	TPA
Endpoint (pure compounds)	EC or PC concentration, lowest effect concentration (e.g. PC10)
Endpoint (mixtures)	Toxic equivalents in pg TEQ/g sample processed
Test duration	24hr (incubation time)
Specificity	Activation of the AP1 pathway only. Ligand selections can be made through compound class selective workup methods and/or metabolic modules.
Assay interferences	Minimal because of use of highly pathway specific construct, and extensive QA/QC. Cytotoxicity and non-specific luciferase interferences experienced with certain ligands and samples can be assessed with the cytotox CALUX assay.
Sensitivity (LOD/Q)	Typically in high pg range (matrix- and sample size-dependent)
Matrices	Any type of sample
Sample volume/mass	Matrix- and desired limit of quantification (LOQ)-dependent
Amount of compound	Typically 10 mg. Lower for high potency compound provided in DMSO
Assessment criteria	In house methods, compliant with relevant application/regulations.
SOPs and Guidelines	BDS internal, similar to ER-, and AR CALUX assays
HTS protocol	BDS; see EURL-ECVAM DB-ALM Protocol n° 197 : Automated CALUX reporter gene assay procedure
Key reference	Piersma AH, Schulp SHW, Uibel F, Van Vugt-Lussenburg, B, Bosgra S, Hermesen SAB, Roelofs MJE, Man, H., Jonker, L., Van der Linden, S, Van Duursen MBM, Wolterbeek APM, , Schwarz M, Kroese ED, Van der Burg B. (2013) Evaluation of an alternative in vitro test battery for detecting reproductive toxicants. Reprod. Toxicol. 38,53-64.



## HIF1alpha CALUX®

The HIF1alpha responsive CALUX consists of the human osteosarcoma cell line U2OS, incorporating the firefly luciferase gene coupled to HIF1alpha responsive elements (HIF1alpha REs) as a reporter gene for the presence of HIF1alpha pathway activating ligands. Following activation by these compounds of the HIF1alpha pathway, HIF1alpha binds to HIF1alpha REs. This will lead to expression of proteins that are under normal circumstances associated to HIF1alpha REs - mediated transcription, but also luciferase. After addition of the appropriate substrate for luciferase, light is emitted. The amount of light produced is proportional to the amount of ligand-specific pathway activation, which is benchmarked against the relevant reference compound, the pathway agonist cobaltous(II)chloride and expressed as toxic equivalents (TEQs), or bioanalytical equivalents (BEQs).

Specification	HIF1alpha CALUX
Basal cell line	U2OS
Species	human
Tissue	bone
Positive control	cobaltous(II)chloride
Endpoint (pure compounds)	EC or PC concentration, lowest effect concentration (e.g. PC10)
Endpoint (mixtures)	Toxic equivalents in pg TEQ/g sample processed
Test duration	24hr (incubation time)
Specificity	Activation of the HIF1alpha pathway only. Ligand selections can be made through compound class selective workup methods and/or metabolic modules.
Assay interferences	Minimal because of use of highly pathway specific construct, and extensive QA/QC. Cytotoxicity and non-specific luciferase interferences experienced with certain ligands and samples can be assessed with the cytotox CALUX assay.
Sensitivity (LOD/Q)	Typically in high pg range (matrix- and sample size-dependent)
Matrices	Any type of sample
Sample volume/mass	Matrix- and desired limit of quantification (LOQ)-dependent
Amount of compound	Typically 10 mg. Lower for high potency compound provided in DMSO
Assessment criteria	In house methods, compliant with relevant application/regulations.
SOPs and Guidelines	BDS internal, similar to ER-, and AR CALUX assays
HTS protocol	BDS; see EURL-ECVAM DB-ALM Protocol n° 197 : Automated CALUX reporter gene assay procedure
Key reference	Piersma AH, Schulp SHW, Uibel F, Van Vugt-Lussenburg, B, Bosgra S, Hermesen SAB, Roelofs MJE, Man, H., Jonker, L., Van der Linden, S, Van Duursen MBM, Wolterbeek APM, , Schwarz M, Kroese ED, Van der Burg B. (2013) Evaluation of an alternative in vitro test battery for detecting reproductive toxicants. <i>Reprod. Toxicol.</i> 38,53-64.

## ER stress CALUX®

The endoplasmic reticulum (ER) stress-responsive CALUX consists of the human osteosarcoma cell line U2OS, incorporating the firefly luciferase gene coupled to ESRE responsive elements (ESRE REs) as a reporter gene for the presence of ESRE pathway activating ligands. Following activation by these compounds of the ESRE pathway, Er stress responsive transcription factors bind to ESRE REs. This will lead to expression of proteins that are under normal circumstances associated to ESRE REs -mediated transcription, but also luciferase. After addition of the appropriate substrate for luciferase, light is emitted. The amount of light produced is proportional to the amount of ligand-specific pathway activation, which is benchmarked against the relevant reference compound, the pathway agonist tunicamycin, and expressed as toxic equivalents (TEQs), or bioanalytical equivalents (BEQs).

Specification	ER stress CALUX
Basal cell line	U2OS
Species	human
Tissue	bone
Positive control	tunicamycin
Endpoint (pure compounds)	EC or PC concentration, lowest effect concentration (e.g. PC10)
Endpoint (mixtures)	Toxic equivalents in pg TEQ/g sample processed
Test duration	24hr (incubation time)
Specificity	Activation of ESRE pathway only. Ligand selections can be made through compound class selective workup methods and/or metabolic modules.
Assay interferences	Minimal because of use of highly pathway specific construct, and extensive QA/QC. Cytotoxicity and non-specific luciferase interferences experienced with certain ligands and samples can be assessed with the cytotox CALUX assay.
Sensitivity (LOD/Q)	Typically in high pg range (matrix- and sample size-dependent)
Matrices	Any type of sample
Sample volume/mass	Matrix- and desired limit of quantification (LOQ)-dependent
Amount of compound	Typically 10 mg. Lower for high potency compound provided in DMSO
Assessment criteria	In house methods, compliant with relevant application/regulations.
SOPs and Guidelines	BDS internal, similar to ER-, and AR CALUX assays
HTS protocol	BDS; see EURL-ECVAM DB-ALM Protocol n° 197 : Automated CALUX reporter gene assay procedure
Key reference	Piersma AH, Schulp SHW, Uibel F, Van Vugt-Lussenburg, B, Bosgra S, Hermesen SAB, Roelofs MJE, Man, H., Jonker, L., Van der Linden, S, Van Duursen MBM, Wolterbeek APM, , Schwarz M, Kroese ED, Van der Burg B. (2013) Evaluation of an alternative in vitro test battery for detecting reproductive toxicants. <i>Reprod. Toxicol.</i> 38,53-64.

## cytotox CALUX®

The cytotoxicity responsive CALUX (cytotox CALUX) consists of the human osteosarcoma cell line U2OS, incorporating the firefly luciferase gene coupled to a constitutively active promoter. This leads to a level of luciferase production, and after addition of the appropriate substrate for luciferase, light. Cell toxic compounds repress this signal dose-dependently. This repression can be used as a measure of cytotoxicity, but also other non-specific influences on CALUX assays, and used to exclude non-specific assay interferences. The reference compound used is tributyltin acetate.

Specification	cytotox CALUX
Basal cell line	U2OS
Species	human
Tissue	bone
Positive control	tributyltin acetate
Endpoint (pure compounds)	EC or PC concentration, lowest effect concentration (e.g. PC10)
Endpoint (mixtures)	Toxic equivalents in pg TEQ/g sample processed
Test duration	24hr (incubation time)
Specificity	Repression of luciferase activity to exclude non-specific assay interferences
Assay interferences	Minimal because of use of highly pathway specific construct, and extensive QA/QC. Cytotoxicity and non-specific luciferase interferences experienced with certain ligands and samples can be assessed with the cytotox CALUX assay.
Sensitivity (LOD/Q)	Typically in microgram range (matrix- and sample size-dependent)
Matrices	Any type of sample
Sample volume/mass	Matrix- and desired limit of quantification (LOQ)-dependent
Amount of compound	Typically 10 mg. Lower for high potency compound provided in DMSO
Assessment criteria	In house methods, compliant with relevant application/regulations.
SOPs and Guidelines	BDS internal, similar to ER-, and AR CALUX assays
HTS protocol	BDS; see EURL-ECVAM DB-ALM Protocol n° 197 : Automated CALUX reporter gene assay procedure
Key reference	Van der Linden, SC, von Bergh A, Van Vugt-Lussenburg B, Jonker L, Brouwer A, Teunis M, Krul C and Van der Burg B. (2014) Development of a panel of high throughput reporter gene assays to detect genotoxicity and oxidative stress, Mutation Res.760,23-32.

## PFAS CALUX®

PFAS are known to be able to interfere in thyroid hormone metabolism by e.g., disrupting the transport of the natural ligand thyroxine (T4) by a major transporter protein transthyretin (TTR). The PFAS CALUX® assay uses this important property to measure the effect of the range of PFAS compounds in chemical mixtures, independent of prior knowledge of their structure. The PFAS CALUX consists of a TTR-binding assay in combination with the TRbeta CALUX bioassay. Disruption of TTR binding by T4 is benchmarked against the reference compound PFOA and expressed as PFOA equivalents/g sample processed. Simple sample processing and clean-up is required, allowing successful application to a wide range of matrices.

Specification	PFAS CALUX
Basal cell line	na
Species	human
Tissue	na
Positive control	PFOA
Endpoint (pure compounds)	EC or PC concentration, lowest effect concentration (e.g. PC10)
Endpoint (mixtures)	Toxic equivalents in pg TEQ/g sample processed
Test duration	42hr (total sample processing time)
Specificity	Measurement of PFAS-mediated TTR interference only. Ligand selection is made through compound class selective workup method, and TTR interference.
Assay interferences	Minimal because of use of purified TTR, highly pathway specific read-out.
Sensitivity (LOD/Q)	Total levels, typically in low µg range (matrix- and sample size-dependent)
Matrices	Any type of sample
Sample volume/mass	Matrix- and desired limit of quantification (LOQ)-dependent
Amount of compound	Typically 10 mg. Much lower for high potency compound provided in DMSO
Assessment criteria	In house methods, compliant with relevant application/regulations.
SOPs and Guidelines	BDS internal
HTS protocol	Not available yet
Key reference	Behnisch P, Besselink H, Weber R, Willand W, Huang J, Brouwer A. (2021) Developing potency factors for thyroid hormone disruption by PFASs using TTR-TRβ CALUX® bioassay and assessment of PFASs mixtures in technical products. Environment International 157, 106791

## TTR assay

Several compound classes are known to be able to interfere in thyroid hormone metabolism by disrupting the transport of the natural ligand thyroxine (T4) by a major transporter protein transthyretin (TTR). The TTR assay uses this important property to measure the effect of the range of PFAS compounds in chemical mixtures, independent of prior knowledge of their structure. The TTR assay consists of a TTR-binding assay in combination with the TRbeta CALUX bioassay. Disruption of TTR binding by T4 is benchmarked against the reference compound tetrabromobisphenol A and expressed as tetrabromobisphenol equivalents/g sample processed. Simple sample processing and clean-up is required, allowing successful application to a wide range of matrices.

Specification	TTR assay
Basal cell line	na
Species	human
Tissue	na
Positive control	tetrabromobisphenol A
Endpoint (pure compounds)	EC or PC concentration, lowest effect concentration (e.g. PC10)
Endpoint (mixtures)	Toxic equivalents in pg TEQ/g sample processed
Test duration	42hr (total sample processing time)
Specificity	Measurement of chemical or chemical-mixture-mediated TTR interference only.
Matrices	Any type of sample
Sample volume/mass	Matrix- and desired limit of quantification (LOQ)-dependent
Amount of compound	Typically 10 mg. Lower for high potency compound provided in DMSO
Assessment criteria	In house methods, compliant with relevant application/regulations.
SOPs and Guidelines	BDS internal
HTS protocol	Not available yet
Key reference	Collet B, Simon E, Van der Linden S, El Abdellaoui N, Naderman M, Man HY, Middelhof I, Van der Burg B, Besselink H, Brouwer A. (2019) Validation of a panel of in vitro methods for assessing thyroid receptor $\beta$ and transthyretin transporter disrupting activities. Reproductive Toxicol., 18, 30554-30559



## H295R assay

The in vitro H295R Steroidogenesis Assay (H295R) described in OECD test guideline 456 (OECD, 2011) utilises human adenocarcinoma cell line NCI-H295R. In this cell line, the human steroidogenesis pathway is fully functional. Upon exposure of this cell line to a test compound, the effect on steroidogenesis can be assessed by quantifying the amount of 17 $\beta$ -estradiol (E2) and testosterone (T) produced by the cells and comparing this to cells exposed to vehicle control only. At BDS, the quantification of E2 and T is performed using the AR CALUX and ER $\alpha$  CALUX bioassays. The goal of the assay according to the OECD guideline is to provide a YES/NO answer with regard to the potential of a chemical to induce or inhibit the production of T and E2; however, it is also possible to report quantitative results by determining a lowest observed effect concentration (LOEC).

Specification	H295R assay
Basal cell line	H295R
Species	human
Tissue	adrenal
Positive control	17 $\beta$ -estradiol and testosterone
Endpoint (pure compounds)	EC or PC concentration, lowest effect concentration (e.g. PC10)
Endpoint (mixtures)	Toxic equivalents in pg TEQ/g sample processed
Test duration	48hr (incubation time)
Specificity	Interaction with endogenous steroidogenesis pathways
Matrices	Any type of sample
Sample volume/mass	Matrix- and desired limit of quantification (LOQ)-dependent
Amount of compound	Typically 10 mg. Lower for high potency compound provided in DMSO
Assessment criteria	In house methods, compliant with relevant application/regulations.
SOPs and Guidelines	BDS internal
HTS protocol	Not available yet
Key reference	Ouedraogo G, Alexander-White C, Bury D, Clewell HJ 3rd, Cronin M, Cull T, Dent M, Desprez B, Detroyer A, Ellison C, Giammanco S, Hack E, Hewitt NJ, Kenna G, Klaric M, Kreiling R, Lester C, Mahony C, Mombelli E, Naciff J, O'Brien J, Schepky A, Tozer S, van der Burg B, van Vugt B, Stuard S, Cosmetics Europe (2022) Read-across and new approach methodologies applied in a 10-step framework for cosmetics safety assessment - A case study with parabens. Regul Toxicol Pharmacol. 2022 May 1:105161.



## Agonism/antagonism

All CALUX®-based assays and most other cell-based assays can be run in two different modes, allowing to distinguish between agonists and antagonists. In the antagonistic mode competition with the reference compound is assessed.

## Metabolic modules

All CALUX®-based assays and most other cell-based assays can also be run with additional metabolic fractions. In case of the U2OS- based CALUX assays, this provides a modular system with the option to include phase I or II separately or together, giving insight in the metabolic route (For more details see Van Vugt-Lussenburg B, Van der Lee RB, Man HY, Middelhof I, Brouwer A, Besselink H, Van der Burg B. (2018) Incorporation of metabolic enzymes to improve predictivity of reporter gene assay results for estrogenic and anti-androgenic activity. Reproductive Toxicol., 75:40-48)

## Miscellaneous

BDS has developed integrated solutions for various practical- and regulatory applications (see Applications section). This includes e.g. bioassays additional to its proprietary stable CALUX® assays, as well as advanced analytical chemistry. Please enquire if you have specific needs.

The combined expertise of our professionals and our well-equipped laboratories also allow for a successful execution of contract research projects. These may include any combination of our skills including routine analyses of samples in our service department, development and application of novel (bio)analytical methods and the execution of complex R&D projects guided by our academically trained staff.

Our ample experience in promoting regulatory acceptance of bioanalytical tools and our extensive quality control schemes make us an attractive partner for setting up, guiding, and executing validation studies. Our contract research projects are always carried out flexibly, according to customer needs, while keeping strictly within the predetermined budgetary and time constraints.



**BioDetection Systems**