DB-ALM Protocol n° 197 : Automated CALUX reporter gene assay procedure

Effects on Endocrine System

This protocol describes a reporter gene assays for the detection of nuclear hormone receptor modulation, or the activation of cell signaling pathways, using high throughput screening.

Résumé

The current protocol describes the performance of BioDetection Systems Chemically Activated LUciferase eXpression (BDS CALUX) reporter gene assays in automated high throughput screening of pure compounds. It has been published before in the context of several screening studies (Piersma et al. 2013, van der Burg et al. 2013, van der Burg et al. 2015a, van der Burg et al. 2015b, van Vugt-Lussenburg et al. 2018).

Experimental Description

Endpoint and Endpoint Measurement:

Receptor agonism, antagonism or cell signaling pathway activation (see the section *Annexes*, Table 1), assessed by luminescence.

Endpoint Value:

PC10 value: the concentration where the test compound gives a response that equals 10% of that of the positive control reference compound.

PC20 value: the concentration where the test compound gives a response that equals 20% of that of the positive control reference compound.

Fold induction (FI)1.5: the concentration where the test compound gives a response 1.5-fold above the assay's background signal (DMSO blank).

Experimental System(s):

Assays are based on the human osteosarcoma cell line U2-OS (ATCC HTB-96), or the rat hepatoma H-4-II-E (ATCC CRL-1548) (AhR CALUX only).

Discussion

- The method described here is an adaptation of BDS CALUX reporter gene assays, some of them already formally validated (OECD, 2016). Although the validated assays are identical to the assays used in the current protocol, the experimental protocol used is slightly different, namely on the type of cell culture plates used (384-wells instead of 96-wells plates). Details like detection limits and acceptable standard deviations may therefore differ, while the overall performance of the cell lines (robustness, stability, predictivity) remains the same.
- No ethical issues arise from the use of these immortalized cell lines.
- Equipment: absolute requirements are a laminar flow cabinet, a cell culture incubator and a luminometer.
- For the automated procedure, as used for the **EU-ToxRisk** case studies, additionally a cell washer-dispenser, a liquid handling machine and a stacker coupled to the luminometer are required.
- The cell lines are genetically modified organisms (GMOs). Therefore, a GMO permit is required (Dutch regulations: safety level Microbiologisch Laboratorium Klasse I (ML-I)).
- Training sessions consist of 1 or 2 weeks, depending on the prior experience of the trainee.
- The CALUX assays are performed over the duration of three days; day 1: seeding; day 2: exposure; day 3: harvesting and measurement.
- The CALUX assays are very sensitive; especially the nuclear receptor hormone assays and the aryl hydrocarbon receptor assay can suffer from background signals caused by e.g. plasticizers leaching from plastics, or hormones present in fetal calf serum. Therefore it is recommended to use only BDS-approved brands of plastic consumables, and to use only BDS-approved dextran coated charcoal (DCC)-stripped fetal calf serum.

Status

In Development:

All assays have passed their developmental phase and have already been used in several high throughput screening programs (Van der Burg 2010b, van der Burg 2015b, Piersma 2013, Sonneveld 2011).

Known Laboratory Use:

CALUX assays are marketed by BDS. As such, many assays are in use in other laboratories (see the section *Annexes*, Table 1, column 'external use'). The method has been used in the **EU-ToxRisk** context.

Participation in Evaluation Studies:

All assays have undergone internal validation with respect to robustness and stability, and predictiveness with respect to known positive controls.

Participation in Validation Studies:

Various CALUX assays have undergone validation studies, and/or participated in inter-laboratory ring studies (see the section *Annexes*, Table 1, column 'validation').

Currently, two assays are in OECD Test Guideline 455 (OECD, 2016), four more assays are being evaluated according to OECD-guidelines, and one assay is ISO-certified (see for further information **http://www.biodetectionsystems.com/fileadmin/user_upload/_temp_/L401-scope_2017-2018_eng.pdf**). Although the validated assays are identical to the assays used in the current protocol, the experimental protocol used is slightly different: the current protocol describes automated 384-wells screening, while the validation was performed manually on 96-wells plates. Details like detection limits and acceptable standard deviations may therefore differ, while the overall performance of the cell lines (robustness, stability, predictivity) remains the same.

Proprietary and/or Confidentiality Issues

The name CALUX is a registered trademark; CALUX assays are marketed by BioDetection Systems (BDS; **http://www.bds.nl/**).

Health and Safety Issues

General Precautions

Standard laboratory procedures apply. Always use gloves and labcoat; when working with dangerous or unknown compounds, always work in a fume hood. If the dilution/exposure work is performed by a liquid handling machine, the risk for the employee is greatly reduced.

MSDS Information

In addition to the safety measures regarding the compounds in use, there are no safety measures needed for the performance of this method.

Abbreviations and Definitions

ATP: Adenosine triphosphate

AhR: Aryl hydrocarbon receptor

BDS: BioDetection Systems bv

CALUX®: Chemically Activated LUciferase eXpression

CDTA: Trans-1,2-diaminocyclohexane-N,N,N'N'-tetra acetic acid monohydrate

DCC: dextran coated charcoal

DCC-Stripped FCS: active charcoal-stripped FCS (a process to remove steroids from the serum)

DMEM/F12: Dulbecco's modified eagle medium supplemented with F12

DMSO: Dimethyl sulfoxide

DTT: Dithiothreitol

EDTA: Ethylenediaminetetraacetic acid

FCS: Fetal calf serum

GMOs: Genetically modified organisms

NADPH: b-Nicotinamide adenine dinucleotide phosphate tetrasodium salt

PBS: Phosphate buffered saline

PC: Positive Control

Trypsinate: Enzymatic treatment of cells with trypsin to remove the intercellular and surface attachment resulting in single rounded cells

Subculture: The transfer of a cell suspension into a new culture flask

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Automated CALUX reporter gene assay procedure
DB-ALM Protocol n° 197

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Materials and Preparations

Cell or Test System

Assays are based on the human osteosarcoma cell line U2-OS (ATCC HTB-96), or the rat hepatoma H-4-II-E (ATCC CRL-1548) (AhR CALUX only).
Each cell line is stably transfected with a reporter gene plasmid containing luciferase under control of the responsive elements of a specific receptor (e.g. estrogen receptor) or cell signaling pathway (e.g. NFκB). Activation of the transfected pathway or receptor by a test chemical results in luciferase production, which can be measured as light production by adding the substrate luciferin. Since most nuclear hormone receptors are not endogenously expressed in U2-OS cells, these receptors have been co-transfected when applicable (see the section ***Annexes***, Table 1).

Equipment

Fixed Equipment

- Cell culture incubator (37°C, 5% CO_2)
- Cell washer-dispenser (BioTek EL406)
- Centrifuge
- Freezers (-20 and -80 and liquid nitrogen)
- Inverted phase contrast Microscope
- Laminar flow cabinet
- Liquid handling machine (Hamilton STARlet)
- Luminometer with stacker

Consumables

Several CALUX assays are sensitive to background signals arising from contaminations present in, or leaching from, reagents and consumables. When a specific brand and order number is quoted below, the advised brand and type is preferred. In cases where the brand and order numbers are only indicative, it is preceded by ‘e.g.’.

- 15 ml tubes (Greiner, 188271)
- 384 wells plates (Greiner, 781080)
- 50 ml tubes (Greiner, 210261)
- 96-deepwell plates (Greiner, 780261)
- Adhesive Plate Seal (e.g. Thermo Fisher, 11524794)
- BreathEasy plate seals (Molecular Devices, E1005)
- Brown autosampler Glass conical vials (VWR, BROW153810)
- Cell culture flasks (75 cm2, e.g. Greiner, 658 175)
- Cryovials (e.g. Greiner, 121261)
- Filter units pore size 0.2 micron (e.g. Whatman 10462200)
- Mr Frosty^{TM} container (e.g. Thermo Fisher, 10110051)
- Pipetting tips 1000, 300 and 50 µl (Hamilton, 235904, 235902 and 235966)
- Sterile reservoirs (50ml) (e.g. VWR, 613-1184)
- Sterile serological pipettes 5ml, 10ml and 25ml (Greiner, 606180, 607180 and 760180)

Media, Reagents, Sera, others

- 1M HCl solution (pH meter) (e.g. Sigma, H1758)
- 1M NaOH solution (pH meter) (e.g. Sigma, S5881)
- Adenosine triphosphate (ATP) (e.g. Ducheva Biochemie BV, A 1335.0010)
- Alcohol 70% (e.g. BioSolve, 5210502)
- b-Nicotinamide adenine dinucleotide phosphate tetrasodium salt (NADPH) (e.g. Applichem, A1395,0500)
- Dithiothreitol (DTT) (e.g. Ducheva Biochemie BV, D1309-0025)
- D-Luciferine (e.g. Resem bv, D-luciferin 500mg)
- Dulbecco's modified eagle medium supplemented with F12 (DMEM/F12) with phenol red as pH-indicator (e.g. Thermo Fisher, 11514436)
- Dulbecco's modified eagle medium supplemented with F12 (DMEM/F12) without phenol red (e.g.Thermo Fisher, 11580546)
- Ethylenediaminetetraacetic acid (EDTA) (e.g. Acros, 147855000)
- Fetal calf serum (FCS) (South American origin, Thermo Fisher, 11573397)
- Glucose-6-phosphate (e.g. Biosynth, G-3340)
- Glucose-6-phosphate dehydrogenase type VII (e.g. Sigma, G7877)
- Glycerol (e.g. Baker, 7044)
- Magnesium carbonate hydroxide (e.g. VWR, AAA18070-0B)
- Magnesium sulphate(e.g. Sigma, M7506)
- $MgCl_2$ hexahydrate (e.g. Sigma, M2393)
- Non-essential amino acids (100x) (e.g. Thermo Fisher, 11140-035)
- Penicillin-streptomycin (e.g. Thermo Fisher Scientific, 15070-063; 5000 penicillin units per ml / 5000 µg streptomycin per ml)
- Phosphate buffered saline pH 7.2 (PBS, e.g. Thermo Fisher, 20012-019)
- Rat liver S9 (e.g. MolTox Trinova, 11-105.5)
- Trans-1,2-diaminocyclohexane-N,N,N'N'-tetra acetic acid monohydrate (CDTA) (e.g. Sigma, 32869)
- Tricine (e.g. Sigma, T0377)
- Tris (e.g. Sigma, T1378)
- Triton X-100 (e.g. Sigma, T8787)
- Trypsin (e.g. Thermo Fisher, 27250-042)

Preparations

Media and Endpoint Assay Solutions

Cell Culture Medium (all solutions should be sterile and handled in a sterile environment):
Take a new bottle of DMEM/F12 with phenol red in the laminar flow cabinet.
Add a tube of FCS (41 ml) to the flask DMEM/F12 (7.5%).
Add 5 ml of non-essential amino acids (100x NEAA).
Add 1 ml of penicillin-streptomycin solution.
Store at 4 ^{o} C for maximum 2 months.

Assay Medium (all solutions should be sterile and handled in a sterile environment):
Open a 500 ml bottle of DMEM/F12 without phenol red in the laminar flow cabinet
Add 26.6 ml DCC-FCS (5% v/v).
Add 5 ml of non-essential amino acids (100x NEAA).
Add 1 ml of penicillin-streptomycin solution.
Store at 4 ^{o} C for maximum 2 months.

Trypsin solution:
Trypsin solution should be diluted with PBS containing 0.2 g/L EDTA until the trypsin solution has a concentration of 0.05% trypsin (g/L). Filter-sterilize using a 0.2 micron pore size filter. Store the tubes of trypsin at -20 ^{o} C until use. After thawing, store at 4 ^{o} C for maximum 2 months.

Freezing medium (all solutions should be sterile and handled in a sterile environment):
Open a 500 ml bottle of DMEM/F12 with phenol red in the laminar flow cabinet.
Remove and discard 143ml of DMEM/F12 medium.
Add 5.5 ml of non-essential amino acids (MEM 100x).
Add 100 ml of FCS.
Add 37.5 ml of DMSO.
Mix gently and distribute as 40 ml aliquots in 50 ml Greiner tubes.
Label the plastic tubes containing the freezing medium (preparation date; expiring date (1 year)).
Store freezing medium at -20 °C.

Triton Lysis Buffer:
Dissolve Tris (25mM), DTT (2 mM) and CDTA (2 mM) in demineralized water.
Add 10% (v/v) glycerol and 1% (v/v) Triton® X-100.
Adjust the pH to 7.8 using 1 M HCl and/or 1 M NaOH solutions.
Transfer aliquots of approximately 40 ml into 50 ml tubes.
Store at -20°C for a maximum of 1 year or at 4°C for a maximum of 1 month.

BDS Illuminate Mix:
Dissolve in demineralized (demi) water: tricine (20 mM), magnesium carbonate hydroxide (1 mM), magnesium sulphate (2.7 mM), EDTA (0.1 mM), DTT (1.5 mM) and D-luciferine (539 µM).
After adding the D-Luciferine, the BDS illuminate-mix should be kept in the dark and further preparation may last no longer than 0.5h due to the instability of the compounds used.
Add ATP (5.49 mM).
Adjust the pH to 7.8 using 1 M HCl and/or 1 M NaOH solutions.
Divide the BDS illuminate mix into 100 ml portions in HDPE bottles.
Close and label the bottles.
Store at -20°C for a maximum of 3 months or at -80°C for one year.

Test Compounds

1. Prepare a 0.1M stock of the test compound in DMSO (if not soluble: go down in 0.5 log unit increments until dissolved).
2. Divide into 100 µl aliquots and store at -20°C.
3. Use glass vials only (Brown autosampler vials)!
4. Before use: thaw one aliquot.
5. The liquid handling robot prepares a dilution series in a 96-deep-well plate; the following 14 concentrations (M) with 0.5Log increments are prepared:

   1.0E-04; 3.0E-05; 1.0E-05; 3.0E-06; 1.0E-06; 3.0E-07; 1.0E-07; 3.0E-08; 1.0E-08;

3.0E-09; 1.0E-09; 3.0E-10; 1.0E-10; 0

6. The compound is exposed by the Hamilton STARlet; the deep-well plate containing the dilutions is discarded.
7. If the compound is tested again later (as a duplicate measurement, or on different assays), a fresh aliquot is used. ***EXCEPTION:*** if a compound is only very scarcely available, the deep-well plates with dilution series will be re-used, and stored at -20 ºC, covered with a plate lid.

Note. For volatile compounds, the 96w-dilution plates are covered with a non-breathable seal until exposure. The assay plates containing the cells exposed to volatile compounds are covered with a breathable seal immediately after exposure.

Note 2 . Compounds insoluble in DMSO cannot be tested using the routine automated HTS-method, and have to be tested separately. For compounds dissolved in water, PBS or medium, a method is available. Compounds in EtOH, MeOH or other solvents will have to be tested manually on 96-wells plates.

Note 3. No specific protocol for very viscous compounds is in place; they may have to be diluted until they can be pipetted by the robotic system. However, within the EU ToxRisk case studies, compounds with such high viscosity have not been encountered yet (CS1 to 5).

Positive Control(s)

For the positive controls (see table 1), aliquots of 1000x stock solutions in DMSO are frozen at -20ºC. For each experiment, the relevant positive controls are thawed, and diluted by the liquid handling machine together with the test compounds (see the section ***Annexes***, Table 1).

Negative Control(s)

DMSO is used as a negative control for all cell lines. A fresh aliquot of DMSO is used for each experiment.

Method

Test System Procurement

Freezing of cells:

- Prepare 75 cm^2 culture flasks with cells (start 1.5 week in advance).
- Check whether the flasks are approximately 90% confluent before starting the freezing procedure.
- Trypsinize all cells according to the procedure described in the subculturing of cells section (below) including the removal of trypsin.
- Suspend the cells in 10ml growth medium per flask.
- Pool and count the cells from the different flasks. Calculate how many cryovials can be prepared at a concentration of 1.5 x 10^6 cells/vial.
- Divide the cells over 50 ml Greiner tubes and centrifuge (250 g, 5 minutes).

> ***Note.*** After the centrifugation step, keep the vials and 50 ml Greiner tubes on ice at all times and work as quickly as possible to minimize the toxic effect of DMSO at room temperature!

- Discard medium and re-suspend the cells in freezing medium. The volume in ml of freezing medium to be used equals the number of vials that can be prepared. Each cryovial should contain 1 ml of cell suspension.
- Divide the re-suspended cells in freezing medium over the number of cryovials calculated (1 ml per cryovial) and close the cryovials.
- Put the cryovials in the Mr. Frosty freezing containers and place it at -80 °C (only overnight) to achieve acooling rate of 1 °C/minute.
- Transfer cryovials to liquid nitrogen for storage.

Thawing of cells:

- Heat water bath to 37°C.
- Take a flask of growth medium from the refrigerator and heat it in the water bath.
- Retrieve a cryovial of CALUX cells from the liquid nitrogen.

> ***Note.*** Wear safety glasses and protective gloves.

- Thaw the cells quickly by gently moving the vial in a water bath of 37°C until the ice has almost melted.
- Clean the outside of the cryovial with 70% alcohol.
- Pipette 0.5 ml of growth medium from the culture flask into the cryovial using a sterile pipette.
- Transfer cells to a 15 ml sterile plastic tube.
- Add drop wise 10 ml of cold cell culture medium and mix gently (4°C).
- Spin down the cells in the centrifuge at approximately 250x g, 5 minutes.
- Remove the medium.
- Resuspend the pellet in 10 ml of culture medium (RT to 37°C), transfer to a culture flask (75 cm^2), and transfer to the CO_2-incubator. Indicate the type of cells, date of preparation, name and passage number.

Routine Culture Procedure

Subculturing of cells:

- Subculture preferably every Monday and Friday.
- Thaw a tube of trypsin.
- Take a flask growth medium from the refrigerator.
- After pre-warming the solutions in a 37ºC water bath, place the trypsin tube and the growth medium and a PBS flask in the safety-cabinet and open them.
- Take a culture flask (75 cm^2) with cells to be subcultured from the CO_2 incubator.
- Transfer the growth medium from the culture flask into the waste bottle by sterile pipetting.
- Carefully pipette 5 ml of PBS into the culture flask by sterile pipetting. Place the tip of the pipette just below the neck of the culture flask. Ensure that the pipette-tip does not to touch the neck of the culture flask.
- Swirl the culture flask approximately 5 times.
- Transfer the PBS from the culture flask into the waste bottle using a sterile 10 ml pipette.
- Rinse again with 5 ml PBS.
- Pipette 2 ml of trypsin into the culture flask by sterile pipetting.
- Swirl the culture flask approximately 5 times.
- Transfer all the trypsin from the culture flask into the waste bottle by sterile pipetting.
- Gently slap the bottom of the culture flask against the palm of your hand after 5 minutes to see if the cells are detaching from the bottom of the culture flask.
- Pipette sterile 10 ml of growth medium supplemented with FCS in the culture flask using a new sterile pipette.
- Swirl the culture flask 5 times allowing the cells to go into suspension. Ensure the cells just below the neck of the flask are in suspension too.
- Re-suspend the cells by performing 10 careful up and down cycles in growth medium using 10 ml pipette. Ensure no flocks of cells are visible anymore.
- Transfer the proper amount of cell suspension into the new culture flask (75 cm^2). The amount of cells to be transferred depends on the growth-rate of the cells and the number of days until the day it is intended to subculture them. Generally, a ratio of 1:6 or 1:8 is appropriate.
- After transfer of cell suspension, fill up the new culture flasks to a final volume of 10 ml with growth medium.
- Label the culture flasks. Indicate the type of cells, date of subculturing, passage number, dilution factor and your name.
- Place the culture flask in the CO2 incubator.

Test Material Exposure Procedures

- Prepare a cell suspension of 1×10^5 cells/ml in white assay medium (see above), and seed white 384-wells plates with 30 µl cell suspension/well using an automatic multidispense multichannel pipette.
- 24 h after seeding, (or, in the case of assays BDS21a and BDS21b, 48h after seeding, see below), prepare exposure medium: prepare a dilution series in 0.5log unit increments of each test compound (in DMSO), in 96W-deepwell plates, and add 1 µl of each concentration to a 96-wells plate containing 500 µl assay medium (or, in the case of assays BDS21a and BDS21b, 10 µl of each concentration, see below).
- Of this exposure medium, add 30 µl, in triplicate, to the assay plates containing the CALUX cells (final DMSO-concentration: 0.1% (or 1% in the case of assays BDS21a and BDS21b, see below)).
- Additionally, prepare DMSO blanks and a full dose-response curve of the relevant reference compounds in similar fashion (for reference compounds and concentrations, see the section ***Annexes***, Table 1).
- At BDS, the preparation of the compound dilution series as well as the exposure of the cells are performed on a Hamilton STARlet liquid handling robot coupled to a Cytomat incubator. This automated CALUX assays have been described in the publication (van der Burg et al. 2015a).

Note 1. In order to be able to detect receptor antagonism, six CALUX assays were also performed in antagonistic mode. The assay procedure was as described above, with the only exception that the cells were supplemented with an EC50 concentration of the reference agonist before exposure (for concentrations and compounds: see the section ***Annexes***, Table 1, column 'EC50 agonist').

Note 2. The assays BDS21a and BDS21b have a slightly modified protocol to ensure good performance. They are seeded 48 h before exposure instead of 24 h; and they are exposed at 1% (DMSO v/v) instead of 0.1%. Additionally, immediately after exposure 6 µl/well of a 10x concentrated S9 mix is added to BDS21b_Geno_RGA_p53S9_act_24h in order to allow metabolic (in)activation by hepatic enzymes. 10x S9 mix consisted of: 3 mg/ml PB-BNF induced Sprague-Dawley rat liver S9, 2 mM NADPH, 30 mM glucose-6-phosphate, 50 mM MgCl2, and 3 units/ml glucose-6-phosphate dehydrogenase. After 3 h, the exposure medium is replaced by assay medium, and cells were allowed to recover for 21 h. After a total of 24 h, cells are harvested and analyzed as described in the following paragraph.

Endpoint Measurement

- After 24 h exposure, remove the exposure medium, e.g. using an EL406 cell washer-dispenser (Biotek).
- Add 10 µl/well triton lysis buffer.
- Measure the luminescent signal in a luminometer (e.g. InfinitePro coupled to a Connect Stacker (both TECAN)), by injecting BDS Illuminate Mix and measuring light output.
- Machine settings:
    - Injection speed: 200 µl/second.
    - Injection volume: 35 µl/well.
    - Measurement time (luminescence): 1 sec/well.
    - Temperature settings: none (ambient).

Acceptance Criteria

Acceptance criteria have been established for triplicate datapoints as well as for the entire 384 wells plates. First, determine for each triplicate on the plate whether it meets the acceptance criteria; then, determine for the entire plate if it meets the acceptance criteria.

- Discard triplicates with a standard deviation >15%
- Discard datapoints where the compound is cytotoxic (<80% cell viability as determined using a separate cytotoxicity assay)
- Using only datapoints that passed the two first criteria: negative control sample (DMSO) should be within set limits (relative induction <10%)
- Using only datapoints that passed the two first criteria: positive control sample (reference compound) maximum induction factor and EC50 value should be within set limits (relative induction >30%; EC50 +/- 0.5 Log units; for EC50 values, see the section ***Annexes***, Table 1)

Perform two analyses as independent (biological) duplicates. If the results (PC10 or PC20 values) of the first and second experiment deviate for >0.5 log unit, perform a third replicate.

Data Analysis

- Average the light counts of the triplicate wells
- Subtract the average blank (DMSO) value
- Set the maximum response elicited by the reference compound at 100% (full receptor- or pathway activation or –inhibition), and scale all other values accordingly.
- Plot the resulting percentage activity (Y) against the compound concentration (X), and fit a sigmoidal curve through the data (e.g. GraphPad Prism Four Parameters, Variable Slope)
- Determine the PC10 concentration, defined as the concentration where the response elicited by the test compound equals 10% of the maximum response of the reference compound. For antagonist- and cytotoxicity experiments, determine PC20 values instead (defined as the concentration where the test compound causes a 20% decrease in the basal signal (set to 100%)).
- Exclude concentrations where the compounds are cytotoxic according to the Cytotox CALUX BDS22a (<80% cell viability) from the analysis.
- For the assays BDS19a (Nrf2 CALUX), BDS15a (TCF CALUX), BDS21a (p53 CALUX) and BDS21b (p53S9 CALUX), determine the concentration where the compound elicits a signal 1.5-fold above the background (DMSO blanks) instead of the PC10 value, since for these assays the reference compounds do not yield a full dose-response curve, and as a consequence no 100% value can be established, and no PC10 can be determined.

Prediction Model

CALUX reporter gene assays are based on U2-OS cells. These cells have very low expression levels of most endogenous receptors, and therefore no cross-talk with other receptors can occur. This makes the assays highly specific for the particular endpoint investigated.

All CALUX assays have undergone a technical validation with known positive and negative compounds. Also, their long-term performance, robustness and stability has been assessed.
Several CALUX assays have been validated internally or externally according to (OECD-) regulations with an elaborate set of reference compounds; literature references are mentioned in the section ***Annexes***, Table 1.
However, for the official validations a different, non-automated 96 wells, protocol was used. In these cases, the general characteristics of the cell lines related to responsiveness, robustness and stability will remain unchanged, but several technical parameters such as detection limits and standard deviations may differ from the 384-wells automated procedure described in the current protocol. All assays have been tested with the automated procedure described here on a database of more than 400 compounds.

Each CALUX assay has a specific toxicological endpoint (see Table 1). CALUX assays have been shown to be applicable for a wide range of applications including, but not limited to, chemicals, cosmetic ingredients, hormones and pharmaceuticals, pesticides, either alone or in complex (environmental) mixtures, like surface and waste water, body fluids, and tissue extracts.

Annexes

Table 1. CALUX assay characteristics. Sources: van der Burg et al. 2010b, van der Burg et al. 2010a, Sonneveld et al. 2011, van der Linden et al. 2014.

Name	Endpoint	EC50 / max	Stock(M)	Reported	Reference compound	EC50 agonist (M)	Validation	Ext. use
BDS1a_EDC_ RGA_ERa_ago_24h	Estrogen receptor α agonism	6.00E-12	1.00E-06	PC10	estradiol		TG455/ van der Burg et al, 2010a	yes
BDS1b_EDC_ RGA_ERa_anta_24h	Estrogen receptor α antagonism	2.40E-08	1.00E-03	PC20	tamoxifen	Estradiol 6E-12	TG455/ van der Burg et al, 2010a	yes
BDS6a_EDC_ RGA_ERb_ago_24h	Estrogen receptor β agonism	1.60E-10	1.00E-05	PC10	estradiol		internal	yes
BDS6b_EDC_ RGA_ERb_anta_24h	Estrogen receptor β antagonism	5.00E-08	1.00E-03	PC20	tamoxifen	Estradiol 2E-10	internal	yes
BDS2a_EDC_ RGA_AR_ago_24h	Androgen receptor agonism	4.50E-10	1.00E-05	PC10	dihydro testosterone (DHT)		OECD in progress / van der Burg et al, 2010b	yes
BDS2b_EDC_ RGA_AR_anta_24h	Androgen receptor antagonism	3.40E-07	1.00E-02	PC20	flutamide	DHT 7E-10	OECD in progress/ van der Burg et al, 2010b	yes
BDS3a_EDC_ RGA_PR_ago_24h	Progestin receptor agonism	3.20E-10	1.00E-05	PC10	Org2058		van der Linden et al, 2014	yes
BDS3b_EDC_ RGA_PR_anta_24h	Progestin receptor antagonism	9.00E-11	1.00E-05	PC20	Ru486	Org2058 3E-10	van der Linden et al, 2015	yes
BDS4a_EDC_ RGA_GR_ago_24h	Glucocorticoid receptor agonism	1.70E-09	1.00E-04	PC10	Dexamethasone (DEX)		internal	yes
BDS4b_EDC_ RGA_GR_anta_24h	Glucocorticoid receptor antagonism	1.40E-09	1.00E-05	PC20	Ru486	DEX 2E-9	internal	yes
BDS5a_EDC_ RGA_TRb_ago_24h	Thyroid receptor β agonism	8.20E-10	1.00E-04	PC10	3,3',5-trioiodo-L-thyronine (T3)		OECD in progress	yes
BDS5b_EDC_ RGA_TRb_anta_24h	Thyroid receptor β antagonism	4.00E-07	1.00E-02	PC20	deoxynivalenol	T3 8E-10	OECD in progress	yes
BDS7a_repro_ RGA_RAR_ago_24h	Retinoic acid receptor agonism	2.00E-07	1.00E-02	PC10	retinoic acid		internal	no
BDS8a_metab_ RGA_LXR_ago_24h	Liver X receptor agonism	1.00E-07	1.00E-03	PC10	GW3965 hydrochloride		internal	no
BDS9a_xenob_ RGA_PXR_ago_24h	Pregnane X receptor agonism	2.60E-07	1.00E-02	PC10	nicardipine		internal	no

Name	Endpoint	EC50 / max	Stock(M)	Reported	Reference compound	EC50 agonist (M)	Validation	Ext. use
BDS10a_metab_ RGA_PPARa_ago_24h	PPARα receptor agonism	2.30E-09	1.00E-04	PC10	GW7647		internal	yes
BDS11a_metab_ RGA_PPARg_ago_24h	PPARγ receptor agonism	6.70E-08	1.00E-02	PC10	rosiglitazone		internal	yes
BDS12a_metab_ RGA_PPARd_ago_24h	PPARδ receptor agonism	1.00E-07	1.00E-02	PC10	L-165,041		internal	yes
BDS13a_xenob_ RGA_AhR_ago_24h	Aryl hydrocarbon receptor agonism	1.00E-11	1.00E-06	PC10	2,3,7,8-TCDD		ISO	yes
BDS14a_hypox_ RGA_Hif1a_act_24h	Hif1a pathway activation	3.20E-04	5.70E-01	PC10	cobaltous(II) chloride		internal	no
BDS15a_Wnt_ RGA_TCF_act_24h	Wnt pathway activation	3.20E-02	1.20E+01	Fold=1.5	lithium chloride		internal	no
BDS16a_regul_ RGA_AP1_act_24h	Activator Protein 1 pathway act.	7.00E-10	1.00E-05	PC10	TPA (PMA)		internal	no
BDS17a_stres_ RGA_ESRE_act_24h	ER stress pathway activation	3.00E-08	1.00E-03	PC10	tunicamycin		internal	no
BDS18a_stres_ RGA_NFkB_act_24h	NFkB pathway activation	3.00E-10	1.00E-05	PC10	TPA (PMA)		internal	no
BDS19a_OxStr_ RGA_Nrf2_act_24h	Nrf2 pathway activation	1.00E-05	1.00E-01	Fold=1.5	curcumine		van Vugt-Lussenburg et al, 2018	no
BDS20a_Geno_ RGA_p21_act_24h	p21 Pathway activation	1.00E-08	1.00E-04	PC10	actinomycin D		van Vugt-Lussenburg et al, 2019	no
BDS21a_Geno_ RGA_p53_act_24h	p53 pathway activation	1.00E-08	1.00E-04	Fold=1.5	actinomycin D		van Vugt-Lussenburg et al, 2020	yes
BDS21b_Geno_ RGA_p53S9_act_24h	p53 pathway activation (+S9)	3.20E-04	1.00E-01	Fold=1.5	cyclo phosphamide		van Vugt-Lussenburg et al, 2021	yes
BDS22a_Tox_ RGA_cytotox_act_24h	Cytotoxicity	2.00E-07	1.00E-02	PC20	tributyltinacetate		van Vugt-Lussenburg et al, 2022	no

Legend .
Name: name of the assay.
Endpoint : receptor or pathway addressed.
EC50 / **max** : concentration where half-maximal activation is achieved (for assays showing a full dose-response curve), or concentration where **maximum activation** is achieved (for assays not showing a full dose-response curve).
Stock (M) : stock concentration of the reference compound.
Reported: the output parameter of the assay (PC10, PC20 or FI).
Reference compound : name of the reference compound used for the assay. EC50 agonist (M): for antagonist assays, an agonist is added to the assay in order to be able to detect repression of this agonist. The column shows the name and concentration of this agonist.
Validation: how is the assay validated (+ reference)?
External (Ext.) use: is the assay used by third parties in other labs yes/no?

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