

# Validation and regulatory acceptance of bio-based (CALUX) analysis of endocrine disruptors

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## Introduction

A growing number of chemicals enter our environment and cause adverse effect by disrupting various biological processes. Mainly analytical analysis tools and animal tests are embedded in regulatory frameworks to monitor chemicals in the environment. *In vitro* reporter-gene bioassays (e.g. CALUX bioassays) are effect-based bio-analytical tools that respond to the presence of chemicals or chemical mixtures. For regulatory acceptance and implementation of *in vitro* bioassays, formal validation and harmonization is required. Recently, we validated the (anti-)estrogen responsive ER $\alpha$  CALUX bioassay for pure compounds according to OECD guidelines. The (anti-)androgen responsive AR CALUX bioassay is currently being validated for pure compounds according to OECD guidelines by EURL ECVAM. The next level of validation of bioassays is the use of complex mixtures or even environmental samples. Validation of the ER $\alpha$  CALUX bioassay for the determination of the estrogenic potential of water and waste water for adoption as an ISO standard has been started.

## Validation study design

### ER $\alpha$ CALUX bioassay according to OECD guidelines

Purpose	To include the ER $\alpha$ CALUX bioassay in the OECD PBTG TG455 <sup>1</sup>
Lead	BioDetection Systems BV, Amsterdam, the Netherlands
Study design	<ul style="list-style-type: none"> <li>- 3 participating laboratories</li> <li>- Intra- and interlaboratory validation</li> <li>- Agonism and antagonism</li> <li>- 23 (agonism) and 12 (antagonism) recommended test chemicals</li> <li>- Review by OECD VMG-NA</li> </ul>
Status	Completed

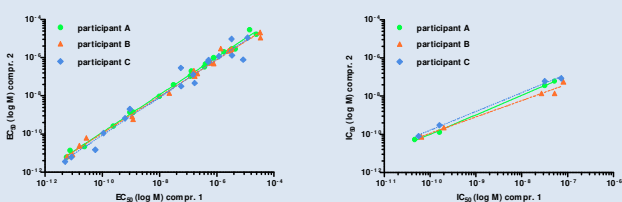
### AR CALUX bioassay according to OECD guidelines

Purpose	To include the AR CALUX bioassay in a new OECD PBTG
Lead	EURL ECVAM, EU Joint Research Center (JRC), Ispra, Italy
Study design	<ul style="list-style-type: none"> <li>- 3 participating laboratories (EU-NETVAL)</li> <li>- Intra- and interlaboratory validation</li> <li>- Agonism and antagonism</li> <li>- 30-50 (agonism) and 30-50 (antagonism) recommended test chemicals</li> <li>- Review by EURL ECVAM and OECD VMG-NA</li> </ul>
Status	Ongoing

### ER $\alpha$ CALUX bioassay according to ISO 19040-3 guideline

Purpose	To include the ER $\alpha$ CALUX bioassay in ISO 19040-3 guideline <sup>2</sup>
Lead	DIN ISO Working Group Genotoxicity and Hormones
Study design	<ul style="list-style-type: none"> <li>- 12 participating laboratories (EU-NETVAL)</li> <li>- Intra- and interlaboratory validation</li> <li>- E1, E2, EE2</li> <li>- 6 water samples; direct exposure</li> <li>- Review by TOXRAT Solutions GmbH, Alsdorf, Germany</li> </ul>
Status	Ongoing

## Results – ER $\alpha$ CALUX validation OECD



**Figure 2** Graphical representation of EC<sub>50</sub> and IC<sub>50</sub> values for all agonistic (left) and antagonistic (right) test compounds, determined following 2 comprehensive studies by each of the participating laboratories. Individual lines represent the correlation between the 2 comprehensive studies.

**Table 15** Average inter-laboratory coefficient of variance (%CV) for the (anti-)ER $\alpha$  CALUX bioassay (agonism and antagonism).

Agonism		Antagonism	
	%CV		%CV
Log[EC <sub>50</sub> ]	3.4	Log[IC <sub>50</sub> ]	1.5
Log[PC <sub>10</sub> ]	3.0	Log[PC <sub>50</sub> ]	2.8

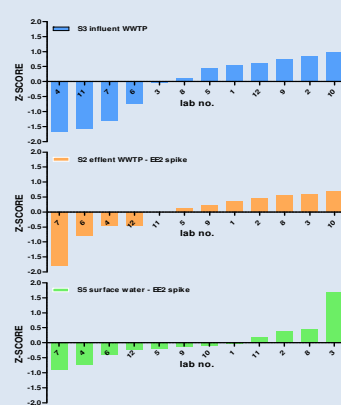
**Table 16** Average inter-laboratory reproducibility variation coefficient (%VC<sub>R</sub>) for the (anti-)ER $\alpha$  CALUX bioassay (agonism and antagonism).

Agonism		Antagonism	
	%VC <sub>R</sub>		%VC <sub>R</sub>
Log[EC <sub>50</sub> ]	2.5	Log[IC <sub>50</sub> ]	1.3
Log[PC <sub>10</sub> ]	2.3	Log[PC <sub>50</sub> ]	1.6

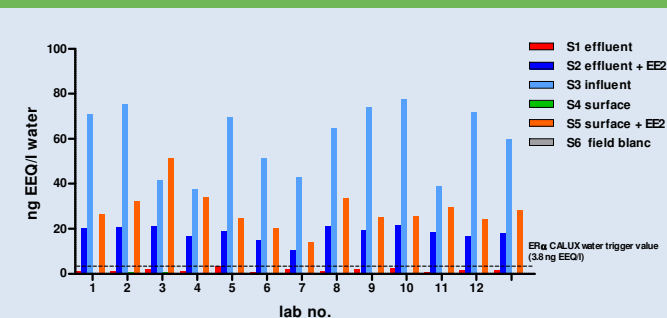
**Table 3** Validation analysis results: concordance of classification (ER $\alpha$  CALUX vs ICCVAM)

Agonism	Lab A	Lab B	Lab D	Antagonism	Lab A	Lab B	Lab D
	Overall accuracy (%)	96	96		100	Overall accuracy (%)	100
Sensitivity (%)	100	100	100	Sensitivity (%)	100	100	100
Specificity (%)	83	83	100	Specificity (%)	100	100	100
False positive (%)	6	6	0	False positive (%)	0	0	0
False negative (%)	0	0	0	False negative (%)	0	0	0
Positive predictivity (%)	94	94	100	Positive predictivity (%)	100	100	100
Negative predictivity (%)	100	100	100	Negative predictivity (%)	100	100	100

## Results – ER $\alpha$ CALUX validation ISO 19040-3



**Figure 2** ISO 19040-3 validation – direct exposure  
 Graphical representation of the z-score for each participating laboratory.  
 At the top, the z-scores for sample 3 (influent WWTP) are given. All participants showed z-scores well between -2 and 2 (-1.6 – 0.98).  
 In the middle, the z-scores for sample 2 (effluent WWTP + EE2 spike) are given. The z-scores varied between -1.8 and 0.68.  
 At the bottom, the z-scores for sample 5 (EE2-spiked surface water) are presented. The z-scores varied between -0.89 and 1.7.



**Figure 2** ER $\alpha$  CALUX analysis results (ng 17 $\beta$ -estradiol eq. (EEQ)/l water) following direct exposure of ER $\alpha$  CALUX cells to effluent (S1), effluent + EE2 spike (S2), influent (S3), surface water (S4), surface water + EE2 spike (S5) or field blank (S6). Analysis results for each participating laboratory are presented. Representing the dotted line, the ER $\alpha$  CALUX drinking-water trigger value as determined by Brand et al.<sup>2</sup> (3.8 ng EEQ/l water), is given.

## Conclusions

### ER $\alpha$ CALUX validation (OECD guideline)

The ER $\alpha$  CALUX bioassay was evaluated and validated for its ability to correctly identify *in vitro* estrogen receptor agonists and antagonists. Inter-laboratory reproducibility varied between 1.3 and 2.5%. Good agonistic accuracy and reliability performance values and very good antagonistic accuracy and reliability performance values were found. The qualitative classification of compounds was in accordance with the ICCVAM classification. The successful validation qualifies the ER $\alpha$  CALUX bioassay for inclusion in the OECD Performance Based Test Guideline TG455.

### AR CALUX validation (OECD guideline)

The AR CALUX bioassay is currently under validation. Participating laboratories have successfully implemented the AR CALUX bioassay for both agonistic and antagonistic analyses. The first set of compounds have been sent to the participants by the coordinator of the study (EURL ECVAM; JRC) and are currently being studied.

### ER $\alpha$ CALUX validation; direct exposure (ISO 19040-3)

Six samples were used for validation of direct exposure of ER $\alpha$  CALUX cells. Results obtained from the 12 participating laboratories showed that z-scores for the test samples were well between -2 and 2 indicating a good (interlaboratory) reproducibility. This shows that direct exposure of the CALUX cells to water samples containing estrogenic activity can be used as alternative for extraction of water samples followed by exposure.

## References

- OECD series on testing and assessment, No. 34. Guidance document on the validation and international acceptance of new or updated test methods for hazard assessment. ENV/JM/MONO(2005)14
- Brand W et al. (2013). Trigger values for investigation of hormonal activity in drinking water and its sources using CALUX bioassays. *Environ Int.* 2013, 55:109-18.



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