European Commission

ESAC Opinion

on the

Scientific Validity of the AR-CALUX® Test Method

ESAC Opinion No. 2019-02
of 5 June 2019

Joint Research Centre

EUR 30272 EN

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The independent scientific peer review of the AR-CALUX® *in vitro* test method described in this report was organised by the Joint Research Centre's EU Reference Laboratory for alternatives to animal testing (EURL ECVAM) and conducted by the EURL ECVAM Scientific Advisory Committee (ESAC).

The ESAC peer review was coordinated by João Barroso on behalf of JRC / EURL ECVAM.

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EU Science Hub
https://ec.europa.eu/jrc

JRC120845

EUR 30272 EN

PDF ISBN 978-92-76-19931-1 ISSN 1831-9424 doi:10.2760/885798

Luxembourg: Publications Office of the European Union, 2020

How to cite this report: EURL ECVAM Scientific Advisory Committee. *ESAC Opinion on the Scientific Validity of the AR-CALUX® Test Method*. EUR 30272 EN, Publications Office of the European Union, Luxembourg, 2020, ISBN 978-92-76-19931-1, doi:10.2760/885798, JRC120845. Available at: http://publications.jrc.ec.europa.eu/repository/handle/JRC120845.

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DIRECTORATE-GENERAL
JOINT RESEARCH CENTRE
Directorate F - Health, Consumers and Reference Materials
European Union Reference Laboratory for Alternatives to Animal Testing (EURL ECVAM)

EURL ECVAM
SCIENTIFIC
ADVISORY
COMMITTEE
(ESAC)

ESAC OPINION

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Scientific Validity of the AR-CALUX® Test Method

ESAC Opinion Nr.	2019-02
Relevant ESAC Request Nr.	2019-02
Date of Opinion	05/06/2019

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Abstract

ESAC, the EURL ECVAM Scientific Advisory Committee, advises EURL ECVAM on scientific issues. Its main role is to conduct independent peer review of validation studies of alternative test methods and to assess their scientific validity for a given purpose. The committee reviews the appropriateness of study design and management, the quality of results obtained and the plausibility of the conclusions drawn. ESAC peer reviews are formally initiated with a EURL ECVAM Request for ESAC Advice, which provides the necessary background for the peer-review and establishes its objectives, timelines and the questions to be addressed. The peer review is normally prepared by specialised ESAC Working Groups. ESAC's advice to EURL ECVAM is formally provided as 'ESAC Opinions' and 'Working Group Reports' at the end of the peer review. ESAC may also issue Opinions on other scientific issues of relevance to the work and mission of EURL ECVAM but not directly related to a specific alternative test method.

The ESAC Opinion expressed in this report relates to the peer-review of the AR-CALUX® *in vitro* test method.

Ispra, 5 June 2019

ESAC Opinion

In 2014, the EU Reference Laboratory for alternatives to animal testing (EURL ECVAM) launched a validation study of the AR-CALUX® *in vitro* test method with the overall goal, provided the validation study would be successful, of proposing the test method to the Organisation for Economic Co-operation and Development (OECD) to become a Test Guideline (TG). In March 2019, the EURL ECVAM Scientific Advisory Committee (ESAC) (Annex 1) was formally asked by EURL ECVAM to conduct an independent peer review of the AR-CALUX® and the EURL ECVAM coordinated validation study and to provide a scientific opinion on the method's scientific validity. An ESAC Working Group (WG) was established for this purpose (Annex 1). The WG met in May 2019 to deliberate on: (1) the scientific basis and biological relevance of the method, (2) its overall performance as assessed during the validation study and (3) its applicability and limitations. Based on its independent assessment, the ESAC WG delivered a detailed peer review report (Annex 2) to support the development of this opinion. The analysis and conclusions of the ESAC WG were based primarily on the EURL ECVAM Validation Study Report, including all the relevant study Annexes and supporting documents. The assessment also included direct requests from the ESAC WG to the test developer for supporting information.

At its 45th meeting, held on 3-5 June 2019 at EURL ECVAM, Ispra, Italy, the non-Commission members of ESAC unanimously endorsed this opinion. Based on the available information, the existing scientific literature and the experts' own extensive experience as detailed in the ESAC WG report, the ESAC unanimously concluded the following:

The AR-CALUX® is a scientifically sound and biologically relevant method for the evaluation of androgen receptor agonism and antagonism

The AR-CALUX® is designed to capture one type of endocrine activity, specifically, binding to the androgen receptor, coupled to the receptor dimerisation and followed by DNA activation, thus covering the molecular initiating event for androgen-mediated adverse outcomes. This assay evaluates both agonism and antagonism. The readout of the assay is by luciferase activation, via DNA transcription. The principles of reporter gene (transactivation) assays and the luciferase response are well established (documented) and accepted by the scientific and regulatory communities. Similar assays have been adopted into OECD Test Guidelines (e.g., Test Guidelines No. 442D, 455, 457 and 458) and the latter three Test Guidelines are included in guidance on assessment of endocrine disruption (OECD Guidance Document No. 150).

The performance characteristics of the AR-CALUX® are fit for the regulatory purpose(s) for which they are defined

Within- and between-laboratory reproducibility was evaluated with twenty chemicals, all tested in four laboratories. The reproducibility of the assay for androgen receptor agonism and antagonism was ≥94% within laboratories and 100% between laboratories.

Twenty-three chemicals could be used to evaluate concordance of the AR-CALUX® predictions with the reference classifications published by the Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM). Based on the positive or negative classification, as defined in the study protocols, the concordance was excellent. For agonism, positive concordance was >90%, negative concordance was 100% and overall concordance was >93%. For antagonism, all parameters were 100%. These performance characteristics demonstrate a robust test method for categorisation of agonism and antagonism of the androgen receptor for regulatory purposes. In line with the Standard Operating Procedure, a number of concentration-based parameters, e.g., EC_{50}, PC_{10}, were calculated, and the former were used to evaluate coefficient of variation for the assay, demonstrating good reproducibility (see additional note below).

The AR-CALUX® is generally applicable to the determination of androgen receptor agonism and antagonism of test chemicals

The AR-CALUX® is ready for screening purposes, mechanistic studies and hazard assessment, as well as for generating supporting information for regulatory prioritisation and decision-making. The ESAC also considers that the assay is suitable for high-throughput screening (HTS) use.

The validation set of forty-six compounds is larger than usually employed and expands on available data from validation of other ARTAs. The chemicals selection was supported by the OECD Validation Management Group Non-Animal (VMG-NA) and the International Cooperation on Alternative Test Methods (ICATM).

The addition of the application of specificity testing using a comparative R^2 rule is considered by the ESAC as a useful additional pragmatic statistical tool to distinguish between true and false antagonists in the manner used in this validation study.

Expansion to other areas of use (e.g., testing of nanomaterials, medical devices) may entail suitable extension of the validation procedures, with respect to the predefined performance criteria.

Conclusion and Recommendation

The ESAC concludes that the AR-CALUX® is a robust test method for categorisation of agonism and antagonism of the androgen receptor. The test method is ready for screening purposes, mechanistic studies and hazard assessment, as well as for generating supporting information for regulatory prioritisation and decision-making.

Additional note on using concentration-response in *in vitro* assays:

The determination of the predictive capacity of *in vitro* assays is often done by dichotomous categorisation of positives and negatives. The assay data, however, often contain a wealth of concentration-response information, which allows (relative) potency assessment of test compounds. Potency information is essential input for the use of assay data in approaches towards quantitative hazard and risk assessment, such as Integrated Approaches to Testing and Assessment (IATA), Defined Approaches (DAs), quantitative Adverse Outcome Pathways (AOPs), Quantitative Structure-Activity Relationships (QSARs), and *in silico* modelling. As *in vitro* methods continue to be developed and validated, it is advisable that possibilities are sought for maximising the use of the concentration-response data in the assessment of relevance of *in vitro* assays.

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Annex 1

COMPOSITION OF THE ESAC AND ESAC WORKING GROUP

Composition of the ESAC and ESAC Working Group

EURL ECVAM Scientific Advisory Committee (ESAC)

Core Members

- Dr. Chantra ESKES (ESAC Chair)
- Prof. Paula M. ALVES
- Dr. Rebecca CLEWELL
- Prof. Emanuela CORSINI
- Prof. Ian COTGREAVE
- Prof. Annette KOPP-SCHNEIDER
- Dr. José Maria NAVAS ANTÓN
- Prof. Aldert PIERSMA
- Dr. Carl WESTMORELAND

Ad-hoc Members

- Dr. Miriam JACOBS

ESAC Working Group (WG)

- Prof. Ian COTGREAVE (WG Chair)
- Dr. Rebecca CLEWELL (WG Rapporteur)
- Prof. Annette KOPP-SCHNEIDER
- Dr. José Maria NAVAS ANTÓN
- Prof. Aldert PIERSMA
- Dr. Miriam JACOBS

Observer

- Dr. Hajime KOJIMA (JaCVAM)

EURL ECVAM (Secretariat)

- Dr. João BARROSO (ESAC Coordinator)
- Dr. Anne MILCAMPS
- Prof. Maurice WHELAN (Head of Unit)

Annex 2

ESAC WORKING GROUP REPORT

EUROPEAN COMMISSION
DIRECTORATE-GENERAL
JOINT RESEARCH CENTRE
Directorate F - Health, Consumers and Reference Materials
European Union Reference Laboratory for Alternatives to Animal Testing (EURL ECVAM)

EURL ECVAM
SCIENTIFIC
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COMMITTEE
(ESAC)

ESAC Working Group Report

on the

Scientific Validity of the AR-CALUX® Test Method

Title page information	
File name	ESAC_WG_Report_AR-CALUX.docx
Abbreviated title of ESAC request	AR-CALUX®
Relating to ESAC REQUEST Nr.	2019-02
Request discussed through	Written procedure following ESAC44 (December 2018)
Report to be handed over to ESAC Chair and EURL ECVAM Coordinator by	Ian Cotgreave (Working Group Chair)

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ESAC Working Group

Full title: ESAC Working Group on the AR-CALUX® Test Method

Abbreviated title: ESAC WG AR-CALUX®

The ESAC WG was established in March 2019 by written procedure to assist in the production of an ESAC Opinion on the scientific validity of the AR-CALUX® *in vitro* test method to assess the androgenic and antiandrogenic activity of chemicals.

This report was prepared at the request of EURL ECVAM by the "ESAC Working Group AR-CALUX®" (ESAC WG), which was charged with conducting a detailed scientific peer review of the EURL ECVAM coordinated validation study of the AR-CALUX® *in vitro* test method. The basis for the scientific peer review was the EURL ECVAM Request for ESAC Advice approved by the ESAC by written procedure following the ESAC44 plenary meeting of December 2018 (ESAC request 2019-02).

The ESAC WG met at EURL ECVAM on 02-03/05/2019 to conduct its peer review. This ESAC WG Report was endorsed by the ESAC WG on 29/05/2019 and represents its consensus view. The Report was endorsed by the ESAC on 05/06/2019.

The ESAC WG had the following members:

- Prof. Ian COTGREAVE (ESAC Core Member, WG Chair)
- Dr. Rebecca CLEWELL (ESAC Core Member, WG Rapporteur)
- Prof. Annette KOPP-SCHNEIDER (ESAC Core Member)
- Dr. José Maria NAVAS ANTÓN (ESAC Core Member)
- Prof. Aldert PIERSMA (ESAC Core Member)
- Dr. Miriam JACOBS (ESAC Ad-hoc Member)

Observer

- Dr. Hajime KOJIMA (JaCVAM)

EURL ECVAM (Secretariat):

- Dr. João BARROSO (ESAC Coordinator)
- Dr. Anne MILCAMPS

ABBREVIATIONS USED IN THE DOCUMENT

- **AR** Androgen Receptor
- **ARTA** Androgen Receptor Transactivation Assay
- **BLR** Between-laboratory reproducibility
- **ESAC** EURL ECVAM Scientific Advisory Committee
- **ESAC WG** ESAC Working Group
- **EU-NETVAL** European Union Network of Laboratories for the Validation of Alternative Methods
- **EURL ECVAM** European Union Reference Laboratory for Alternatives to Animal Testing
- **GLP** Good Laboratory Practice
- **ICATM** International Cooperation on Alternative Test Methods
- **ICCVAM** Interagency Coordinating Committee on the Validation of Alternative Methods
- **JRC** Joint Research Centre
- **OECD** Organisation for Economic Co-operation and Development
- **QA** Quality Assurance
- **QC** Quality Control
- **SOP** Standard Operating Procedure
- **TG** Test Guideline
- **TGP** Test Guidelines Programme
- **VMG** Validation Management Group
- **VMG-NA** Validation Management Group Non-Animal
- **VSR** Validation Study Report
- **WLR** Within-laboratory reproducibility

1. Study objective and design

1.1 Analysis of the clarity of the study objective's definition

(a) ESAC WG summary of the study objective as outlined in the Validation Study Report

In 2014, EURL ECVAM launched a validation study of the AR-CALUX® method with the overall goal, provided the validation study would be successful, of proposing the test method to OECD to become a Test Guideline (TG). In March 2019, the ESAC was formally asked to provide independent review and provide scientific opinion to EURL ECVAM on the AR-CALUX® method. The ESAC AR-CALUX® Working Group (WG) was formed and met in May 2019 to deliberate on: (1) the scientific basis and biological relevance of the method, (2) its overall performance as assessed during the validation study and (3) its applicability and limitations.

(b) Appraisal of clarity of study objective as outlined in the Validation Study Report

The ESAC agrees that the study objectives are clear in the Validation Study Report (VSR).

1.2 Quality of the background provided concerning the purpose of the test method

The VSR clearly describes the intended application of the AR-CALUX® test method. The regulatory testing purpose of the test method is to screen for human relevant androgen receptor (AR) agonism and antagonism, as part of level 2 of the OECD conceptual framework for the testing and assessment of endocrine disruptors. It is an additional method to the current OECD TG 458, which is also an Androgen Receptor Transactivation Assay (ARTA) (OECD 2016), and the regulatory applications and guidance can be found in OECD GD 150 (OECD 2018).

(a) Analysis of the scientific rationale provided in the Validation Study Report

The assay is designed to capture the downstream events following binding to the AR, subsequent receptor dimerisation, followed by DNA activation, thus covering the molecular initiating event for androgen-mediated adverse outcomes. The readout of the assay is luciferase signal activated via DNA transcription and transactivation of a luciferase reporter construct.

(b) Analysis of the regulatory rationale provided in the Validation Study Report

Internationally, there is a well-recognised regulatory need to provide test systems for the detection of chemicals with endocrine activity, focusing at first on estrogen, androgen, thyroid and steroidogenesis modalities. Whilst *in vitro* assays for estrogenic and steroidogenic modalities have been developed as TGs, the development and validation of androgen receptor and thyroid *in vitro* test methods are taking longer.

The international regulatory need for AR assays was reflected several years ago, by the acceptance of the European Commission (EC) proposal (standard project submission form) to the OECD Working Group of National Coordinators of the Test Guidelines Programme (WNT), to conduct the AR-CALUX® validation as part of the OECD Test Guidelines Programme (TGP). This is clearly reflected in the VSR.

At the time of the project proposal submission, there were no successfully validated ARTA(s), though the AR Ecoscreen, led by Japan, was going through validation. Other AR binding assays were dropped from the TGP, as the lead countries were no longer in a position to continue validation work, and also because there was an international regulatory preference for test methods that included more downstream information. e.g., DNA transactivation. The AR Ecoscreen and AR-CALUX® test methods, both being ARTAs, therefore met a critical gap as identified in the OECD endocrine disruptor testing and assessment work programme. The AR Ecoscreen was successfully validated and peer reviewed in 2015, leading to TG approval and declassification in 2016 (TG 458). The AR-

CALUX® assay validation was already well underway, and provides an additional AR modality assay, also using the luciferase signaling technology. It is therefore suitable to join the current TG 458 with the same regulatory rationale.

1.3 Appraisal of the appropriateness of the study design

The study and data reported in the VSR comply with the principles and criteria set out in the OECD Guidance Document on the Validation and International Acceptance of New or Updated Test Methods for Hazard Assessment (No. 34, OECD, 2005), and described in the generally accepted Modular Approach to validation (Hartung et al., 2004).

Number of laboratories: The number of laboratories was originally three, which is satisfactory. A fourth laboratory was added, as a precautionary measure, when there were concerns regarding the performance of one of the three laboratories early on in the transfer and reproducibility phases of the study. However, this laboratory was able to take appropriate corrective action by deadlines given and completed the reproducibility study. Therefore, the results from the fourth laboratory are included in the final analysis. The fourth laboratory is not essential to the validation, but does augment the data.

Organisation of the study: This study was organised according to standard Joint Research Centre (JRC) procedures using EU-NETVAL laboratories, and the ESAC supports this process.

Chemical selection: The procedure for chemical selection, which was carried out by EURL ECVAM in consultation with the OECD Validation Management Group Non-Animal (VMG-NA) and the International Cooperation on Alternative Test Methods (ICATM) was deemed appropriate by the ESAC. However, with regard to the use of mixtures, e.g., Arachlor 1254, ESAC warns against using ill-defined mixtures in validation studies. In addition, the ESAC recommends that the use of chemicals on the Stockholm Persistent Organic Pollutants (POPs) Convention lists or banned in certain OECD member countries (e.g., Arachlor 1254, PCBs, DDT, methoxychlor) should preferably be avoided in the proficiency chemical set.

Quality Assurance (QA) of data: Good Laboratory Practice (GLP) processes were followed in three laboratories. The fourth laboratory, which is also the test method developer, explained that although they do not conduct this assay to full GLP documented inspection standards in-house, they do have an independent Quality Control (QC) officer, and do follow GLP-like processes (see section 3.1).

Statistician independence: To ensure objectivity, the statistical analysis was conducted in a blinded manner by EURL ECVAM, with no possibility for any subjective judgement.

1.4 Appropriateness of the statistical evaluation

Standard EURL ECVAM approaches were used, with the addition of the application of specificity testing using a comparative R^2 rule to distinguish between true and false antagonists. This addition is considered by the ESAC as a useful pragmatic statistical tool for this validation study. However, it is sufficient to base the R^2 rule solely on correlation. Linear regression is not appropriate in this situation, because both variables are associated with error. This recommendation will neither affect the results of the validation nor the conclusions drawn.

2. Collection of existing data

2.1 Existing data used as reference data

The following sources were used for reference data in the VSR:

- Chemical selection for the validation study: Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM) recommendation list of 2003; AR Ecoscreen results from 2005 (Araki et al., 2005), and from the validation study and TG 458 (OECD, 2016); AR-CALUX® published results (van der Burg et al., 2010) and PALM assay results (Freyberger et al., 2012).
- Assessment of predictive capacity (categorisation): ICCVAM list of 2017 (Kleinstreuer et al., 2017); ARTA of Japan (TG 458); ARTA of Korea (validation study report); Tox21 lux, Tox21 bla; AR-pathway model.
- Comparison of variability:
    - ER-CALUX® (TG 455):
        - For agonist testing (log EC_{50}) (n=17): 1.2% to 3.1% CV
        - For antagonist testing (log IC_{50}) (n=4): 0.5% to 1.6% CV
    - ARTA of Japan (EcoScreen (TG 458 OECD 2016))
        - For agonist testing (log PC_{50}) (n=3): 0.38% to 1.53% CV
        - For antagonist testing (log IC_{50}) (n=3): 0.84% to 1.15% CV

The ESAC considers these data appropriate for their use in this validation study.

2.2 Existing data used as testing data

Not Applicable.

2.3 Search strategy for retrieving existing data

The ESAC supports the search strategy, which was already agreed by the VMG-NA, ICATM and Tox21 partners (see Annex 13.3 of the VSR).

2.4 Selection criteria applied to existing data

The ESAC agrees with the pre-defined criteria applied in data selection (see Annex 13.3 of the VSR). The ESAC also appreciated the transparency of the description of the selection criteria.

3. Quality aspects relating to data generated during the study

3.1 Quality assurance systems used when generating the data

Good *In Vitro* Method Practice (GIVIMP) is not applicable, as it was published in 2018, which was after the completion of validation data collection. Nevertheless, many of the principles of practice were applied in the validation study. Good Cell Culture Practice (GCCP) is generally used in SOP development, but does not need to be specified. Full GLP compliance is not a requirement for test method development or validation. Nonetheless, the use of GLP was ensured in three test facilities and confirmed by appropriate Statements. The fourth laboratory is ISO accredited for two other

methods, and has an in-house QC officer, as well as following GLP-like practices. The ESAC endorses the quality assurance measures taken in this validation study.

3.2 Quality check of the generated data prior to analysis

See Section 3.1. Furthermore, in addition to in-house quality assurance procedures, QA checks were performed at EURL ECVAM.

4. Quality of data used for the purpose of the study (existing and newly generated)

4.1 Overall quality of the evaluated testing data (newly generated or existing)

In the initial transfer phase, several technical problems were incurred, which led to rejection of data based on the predefined acceptance criteria. This was overcome in later phases, with assistance from EURL ECVAM and the test method developer. This resulted in average CVs under 5% in the final predictive test phase, which is considered by the ESAC as excellent.

4.2 Quality of the reference data for evaluating relevance[1]

The biological relevance of the test system is established, and the reference data used for comparison includes established mechanisms for both agonism and antagonism. Comparisons were made with validation reference data from similar androgen receptor stably transfected transactivation assay (STTA) – AR EcoScreen (Japan) and ARTA (Korea), as well as with supplementary sources, including Tox21 AR transactivation assays, and the EPA AR pathway model. The performance values were calculated in comparison to the more recent ICCVAM reference lists. The ESAC considers these comparisons are appropriate.

Comparison with *in vivo* Hershberger data was performed by EURL ECVAM, but is not considered by ESAC to be essential to the final conclusions, because the Hershberger is not considered to be reliable. ESAC suggested comparison of the AR-CALUX® with data with the validated 21 Day Androgenised Female Stickleback Endocrine Screening Assay (OECD, 2011), as well as the Rapid Androgen Disruption Adverse outcome Reporter (RADAR) assay (Sébillot et al., 2014), which is currently in validation. These comparisons were conducted by EURL ECVAM, but the currently available Stickleback and RADAR data are considered too limited to yield a useful comparison at this time.

4.3 Sufficiency of the evaluated data in view of the study objective

The ESAC considers the quality of the entire data set sufficient to draw robust conclusions (see 4.1 and 4.2).

[1] OECD guidance document Nr. 34 on validation defines relevance as follows: "Description of relationship of the test to the effect of interest and whether it is meaningful and useful for a particular purpose. It is the extent to which the test correctly measures or predicts the biological effect of interest. Relevance incorporates consideration of accuracy (concordance) of a test method."

5. Test definition (Module 1)

5.1 Quality and completeness of the overall test definition

With regard to the overall test system, relevance, criteria and protocol, the ESAC considers that:

a) The test system is well described, but there is a lack of information on cell line characterisation. Low phase I, II and III activities have been characterised in the cell line (personal communication with test developers), and using TempoSEQ this is regularly checked and guaranteed on clones provided to end users. The ESAC understands that this information will be included in an updated VSR.
b) The method has high relevance to the activation of the androgen receptor.
c) The criteria were strict and delivered desirable CV characteristics.
d) The SOPs are sufficiently described and several existing VMG recommendations in the revised SOPs are endorsed by the ESAC. ESAC also recommends the use of bold text and cautionary notes for critical elements. These include:

- The preferred use of commercial premixed (10%) stocks of Triton X-100.
- The need to use high purity DMSO. New lots should be tested against the inclusion criteria.
- The need to ensure appropriate temperature of luciferin in the assay.
- The importance of *a priori* checking of glassware and plasticware for performance, considering also effects of chemical contamination during dilution.
- The need for *a priori* testing of new lots of serum for performance as well as mycoplasma contamination.

5.2 Quality and completeness of the documentation concerning SOPs and prediction models

The ESAC supports the use of the SOPs, with the planned revisions by EURL ECVAM.

6. Test materials

6.1 Sufficiency of the number of evaluated test items in view of the study objective

In the view of the ESAC the numbers of test items exceed the minimum acceptable number. Further, the expanded number of chemicals tested in this validation study compared to other ARTA assays is appreciated.

6.2 Representativeness of the test items with respect to applicability

The ESAC considers that the chemical selection process to derive structural diversity was well thought-out and executed with appropriate consultation. Appropriate diversity was evident for the antagonist and negative test sets. It is acknowledged that fewer known agonists are available, and therefore the test set is clustered around the steroid structures. As with any *in vitro* assay, chemicals being difficult to test (Volatile Organic Compounds (VOCs) and certain lipophilics, etc.) are not included in the validation study.

7. Within-laboratory reproducibility (WLR) (Module 2)

7.1 Assessment of repeatability and reproducibility in the same laboratory

The ESAC agrees that this is fit for purpose and considers that good concordance in predictions amongst runs was achieved. Repeatability versus reproducibility was not systematically considered in the study design. However, based on the low CVs, ESAC is confident in the reproducibility.

The lead laboratory, BDS did provide historic control data in response to ESAC questions on historic WLR. These data are satisfactory and in line with ESAC's overall conclusions.

7.2 Conclusion on within-laboratory reproducibility as assessed by the study

The conclusions on within-laboratory repeatability and reproducibility are justified by the data as evaluated.

8. Transferability (Module 3)

8.1 Quality of design and analysis of the transfer phase

The ESAC considers that the design of the transfer phase and the pre-described criteria for the phase were appropriate and effective.

8.2 Conclusion on transferability to a naïve laboratory / naïve laboratories as assessed by the study

Potential critical issues have been identified in the protocols and the SOPs appropriately amended. This supports effective transfer to a naïve laboratory.

9. Between-laboratory reproducibility (BLR) (Module 4)

9.1 Assessment of reproducibility in different laboratories

The BLR was assessed both as %CV (intrinsic data variability) and as concordance of predictions between laboratories. The BLR was considered by the ESAC to be good.

9.2 Conclusion on between-laboratory reproducibility as assessed by the study

The ESAC considers this to be good.

10. Predictive capacity and overall relevance (Module 5)

10.1 Adequacy of the assessment of the predictive capacity in view of the purpose

The ESAC considers the assay as fit for the intended purpose as described in the validation report (see section 4.2).

10.2 Overall relevance (biological relevance and accuracy) of the test method in view of the purpose

The ESAC considers that the assay clearly addresses the androgen receptor mode of action at the cellular level. The assay is relevant for the prediction of a primary component in androgen receptor mediated response and, as such, is relevant for regulatory use. However, it is acknowledged that steroid hormones can be active to differing degrees amongst the steroid receptors, as witnessed by the low activity of progesterone and oestrogen in the test system. This entails that steroids other than androgens will show activity in this assay and is consistent with *in vivo* endocrine biology.

11. Applicability domain (Module 6)

11.1 Appropriateness of study design to conclude on applicability domain, limitations and exclusions

The ESAC considers that the chemical space coverage is more than adequate for the validation and expands on available data from validation of other ARTAs. Indeed, the chemical selection was clearly supported by the OECD VMG-NA throughout. In addition, mapping of the chemical space (based on chemical structural features) demonstrates that the validation chemical selection provided appropriate coverage of the REACH chemical space, as much as can be achieved with the limited number of chemicals that can feasibly be tested in a validation study.

11.2 Quality of the description of applicability domain, limitations, exclusions

The ESAC considers that the applicability domain is sufficiently described with respect to the aims of the validation study.

Expansion to other areas of use, such as testing of nano-materials and medical device materials, may entail suitable extension of the validation procedures, with respect to the predefined performance criteria.

As identified in other validation studies using the luciferin/luciferase reporter system, the use of some phytochemicals, such as genistein and daidzein, can non-specifically augment the signals reported in the luminometer, leading to false positives.

12. Performance standards (Module 7)

12.1 Adequacy of the proposed Essential Test Method Components

Not Applicable.

12.2 Adequacy of the proposed Reference Chemicals

Not Applicable.

12.3 Adequacy of the proposed performance target values

Not Applicable.

13. Readiness for standardised use

13.1 Assessment of the readiness for regulatory purposes

The ESAC considers that the assay is ready for regulatory purposes, as all requirements have been met during validation.

13.2 Assessment of the readiness for other uses

The AR-CALUX® is also ready for screening purposes, mechanistic studies and for supporting information for hazard/risk assessment.

13.3 Critical aspects impacting on standardised use

The ESAC considers that the assay is suitable for high-throughput screening (HTS) use (van der Burg et al., 2015). The ESAC have seen letters between ATCC and BDS from 2002 and are satisfied with respect to intellectual property (IP) considerations and permissions for use. Fair, Reasonable, and Non-Discriminatory (FRAND) conditions will need to be applied (OECD, 2019).

13.4 Gap analysis

The current description of the model lacks a full characterisation of the presence of the relevant hormone-dependent receptors and down-stream regulators in the test system that may affect the read-out. However, the ESAC acknowledges the authentication of the cell line, as performed by the JRC. In addition, the ESAC considers the low expression of the glucocorticoid receptor/mechanism in the AR-CALUX® cells to be important in minimising potential receptor cross-talk in the system.

14. Other considerations

A clearer (GANTT) indication of the time-lines associated with each phase of the validation could be provided.

The first classifier utilises “positive”, “negative” and “inconclusive” categories. ESAC agrees with the change proposed by the VMG, to use a classifier using only two categories, “positive” and “negative”. ESAC also agrees to the use of the interpolation approach, to predict the value at a dilution factor of 2, to re-evaluate the validation data and provide proof of concept for the final SOP (with a dilution factor of 2). This is an appropriate adaptation in response to the validation data.

15. Conclusions on the study

15.1 ESAC WG summary of the results and conclusions of the study

With the recommendations provided, this assay is considered ready to be taken forward to Test Guideline development.

15.2 Extent to which study conclusions are justified by the study results alone

Based on the appraisal performed by the ESAC, the study conclusions are clearly justified by the study results alone.

15.3 Extent to which conclusions are plausible in the context of existing information

Considering the lack of chemicals with established androgenic properties, the chemical coverage utilised in the validation was commendable.

16. Recommendations

16.1 General recommendations

It is appreciated that, subsequent to the validation study, the AR-CALUX® assay has been expanded to include aspects of metabolic capacity (van Vugt-Lussenburg et al., 2018), in concordance with the recommendations of the OECD detailed review paper (DRP) 97 (OECD, 2008). This could potentially be used to extend biological relevance of the test for chemicals that are activated or inactivated via metabolism.

The determination of the predictive capacity of *in vitro* assays is often done by dichotomous categorisation of positives and negatives. The assay data, however, often contain a wealth of concentration-response information, which allows (relative) potency assessment of test compounds. Potency information is essential input for the use of assay data in approaches towards quantitative hazard and risk assessment, such as Integrated Approaches to Testing and Assessment (IATA), Defined Approaches (DAs), quantitative Adverse Outcome Pathways (AOPs), Quantitative Structure-Activity Relationships (QSARs), and *in silico* modelling. As *in vitro* methods continue to be developed and validated, it is advisable that possibilities are sought for maximising the use of the concentration-response data in the assessment of relevance of *in vitro* assays.

16.2 Specific recommendations (e.g. concerning improvement of SOPs)

In addition to the specific recommendations provided by the VMG, and provided in section 5.1 above, eventual use of drying ovens in glass-ware re-use should account for any potential contaminations from the plastic/rubber linings and other fixtures in the oven. This extends to consideration of potential contamination from all lab-ware, and from the local laboratory environment (building material, air, water, etc.). The test users should also avoid the use of glassware with any protective coatings. Finally, including potency in data interpretation (such as with IC_{50} or PC_{10} data calculated in this validation study) would improve and facilitate broader (regulatory) use of the data generated by the assay (see section 16.1).

17. References

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KJ-NA-30272-EN-N

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doi:10.2760/885798

ISBN 978-92-76-19931-1