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Full length article



ReadEDTest: A tool to assess the readiness of *in vitro* test methods under development for identifying endocrine disruptors

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ABSTRACT

Growing evidence shows that endocrine disruptors (EDs), known to affect the reproductive system, may also disturb other hormone-regulated functions leading to cancers, neurodevelopmental defects, metabolic and immune diseases. To reduce exposure to EDs and limit their health effects, development of screening and mechanism-based assays to identify EDs is encouraged. Nevertheless, the crucial validation step of test methods by regulatory bodies is a time- and resource-consuming process. One of the main reasons of this long duration process is that method developers, mainly researchers, are not fully aware of the regulatory needs to validate a test. We propose an online self-assessment questionnaire (SAQ) called ReadEDTest easy to be used by all researchers. The aim of ReadEDTest is to speed up the validation process by assessing readiness criteria of *in vitro* and fish embryo ED test methods under development. The SAQ is divided into 7 sections and 13 sub-sections containing essential information requested by the validating bodies. The readiness of the tests can be assessed by specific score limits for each sub-section. Results are displayed via a graphical representation to help identification of the sub-sections having sufficient or insufficient information. The relevance of the proposed innovative tool was supported using two test methods already validated by the OECD and four under development test methods.

1. Introduction

The chemical exposome, representing all the substances the humans are exposed to along their life from conception to death, is highly associated with severe health outcomes (Wild, 2005). Among these substances present in everyday products, some have been linked to endocrine disruption (i.e. mimicking or interfering with hormones), and are associated with increased risks for environment and human health such as infertility, neurodevelopmental disorders or metabolic diseases (Cediel-Ulloa et al., 2022; Heindel et al., 2022; Heindel et al., 2017). These chemicals are defined as endocrine disruptors (EDs). Due to their

ubiquity and their putative adverse effects, the European Commission is considering reinforcing their regulation by submitting them to a generic approach to risk management, as well as adding a new hazard class for EDs in the Regulation (EC) No 1272/2008 on classification, labeling and packaging (CLP) of substances and mixtures (COM(2020) 667 final).

Although some regulatory measures have been proposed in the European Union (EU) and other parts of the world to restrict some ED production and to avoid their spreading, all substances that might perturb the endocrine system have not been identified yet. Even if all existing biological knowledge must be taken into consideration, regulations request methods validated by the OECD (Organization for

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Economic Co-operation and Development) or the EPA (Environmental Protection Agency). Currently available methods are far from representing all biological systems that may be disturbed by these substances. In the current OECD conceptual framework for EDs, chemicals acting on estrogen, androgen, thyroid, steroidogenic (EATS) pathways are partially covered by *ad hoc* test guidelines (TGs) relying on either, *in vitro* or *in vivo* models in mammalian and non-mammalian species (Revised Guidance Document, 2018). Recently, a study has explored the available databases and the scientific literature to identify existing non-validated ED characterization methods that could be prioritized for validation in missing regulatory key areas (Zgheib et al., 2021).

In the EU, the challenge for the regulatory agencies to identify and to reduce exposure to EDs is increasing due to the recent and emerging regulatory changes. Among the measures addressing EDs in the Chemical Strategy for Sustainability are the development of new and effective test methods for ED identification (Communication from the commission to the European parliament, 2020). Those tests could further be part of a coherent integrative testing strategy (ITS) which could be in an integrated approach to testing and assessment (IATA) (Grignard et al., 2022).

In the EU, the recognition of the lack of methods has led to the funding of research programs such as the EURION cluster to Improve Identification of EDs (<https://eurion-cluster.eu/>) (Street et al., 2021). This cluster, composed of eight independent H2020 projects funded by the EU, was formed in 2019 to jointly develop and improve testing methods to identify EDs regarding major outcomes (metabolism disruption, female infertility, neurodevelopmental defects, etc...) to be integrated in testing strategies, as well as to Adverse Outcome Pathways (AOP) to support regulatory decisions under a Weight of Evidence approach (Bajard et al., 2022). The H2020 OBERON project (<http://oberon-4eu.com/>) (Audouze et al., 2020), which is part of EURION, aims to develop a battery of new alternative test methods for identification of EDs related to metabolic disorders using the concept of IATA (Street et al., 2021).

In 2022, the European Partnership for the Assessment of Risks from Chemicals (EU Horizon PARC project) proposes a broader approach for the next seven years, directed towards the development of NAMs (New Approach Methodologies), involving *in vitro*, *in vivo* and *in silico* methodologies, concerning the human and the environmental impacts, which include EDs.

Other international research and validation efforts have been made with the support of OECD dedicated programs (OECD Work Related to Endocrine Disruptors, <https://www.oecd.org/env/ehs/testing/oecdworkrelatedtoendocrinedisruptors.htm>), and of the United States (US) Environmental Protection Agency (EPA) with the Endocrine Disruptor Screening Program (EDSP, <https://www.epa.gov/endocrine-disruption/endocrine-disruptor-screening-program-edsp-overview>).

The validation of test methods, preliminary to their use in a regulatory context, is a long process (several years on average) involving several crucial steps to demonstrate their transferability through a standard operating protocol, their repeatability and reproducibility, their specificity and sensitivity in various laboratories belonging, ideally, to different OECD member countries. All these information can be found in the OECD Guidance document number 34 (<https://ntp.niehs.nih.gov/iccvam/suppdocs/feddocs/oecd/oecd-gd34.pdf>). Ultimately, the validation process results in a draft TG that is subjected to peer-reviewing process within *ad hoc* expert working groups and public commenting rounds before its potential adoption as a TG by the Working Group of National co-ordinators of the Test Guidelines programme (WNT). Other reasons for this duration are linked to the operational and financial supports, that are needed to implement the operational steps in the validation, and to insufficiently mature methods at the start of the validation process. Hence, the validation process appears inherently long, but could be speed up if some critical and essential information were already fulfilled by the test developers before submitting their assay to this program.

Regarding the non-validated test methods, it is therefore necessary to bring them to a sufficient level where they meet all the necessary criteria to be later implemented in the regulatory framework. Recently, the French Pepper platform was established to help accelerating the validation process for methods regarding ED identification (Fig. 1).

The role of Pepper is to identify test methods under development, and to accelerate the passage from a method developed in an academic environment to a method that can be used in a regulatory context. In a first study, in collaboration with several partners including Inserm, various sources of information were investigated (literature, survey and calls for submissions) to decipher which methods are filling gaps in the set of validated tools available for regulatory assessment, and which ones are mature enough to enter a validation process (Zgheib et al., 2021). Among the identified methods, some were selected by the Pepper's Relevance Committee and several test laboratories for inter-laboratory studies were selected. As a next step, transferability studies, where all "naive labs" implement the methods on known substances, are done and they often reveal and correct weaknesses in the procedures. Then, a ring test is organized, with blind coded substances in order to check reproducibility, reliability, applicability domain of the methods on a large set of substances, that lead to a validation report. Along this process, Pepper is assisted by an international Validation Management Group, supporting the acceleration of the validation process.

Moreover, Pepper helps to connect with the OECD process to develop the TG by submitting to the WNT, responding to peer reviews and elaborating the TG.

To speed up the process of validation, we developed an online tool called ReadEDTest that evaluates the readiness of *in vitro* (cell-based) and alternative *in vivo* ED characterization test methods under development. ReadEDTest is a self-assessment questionnaire (SAQ) easy to use by all researchers, that lists essential information (scientific and related to a potential regulatory use) requested by the validating bodies. At the end of the SAQ, based on a battery of criteria, a score is generated to help a direct evaluation of the strengths and weaknesses of the method under development. It will therefore give the information whether the test is ready for validation or not. ReadEDTest is supported by two OECD validated methods and has been evaluated with four *in vitro* methods under development in the OBERON project. ReadEDTest is accessible via a web interface at <https://readedtest.u-paris-sciences.fr/> (Fig. 2).

2. Material & methods

2.1. Structure of the self assessment questionnaire (SAQ)

The SAQ helps researchers to assess the readiness of the *in vitro* and zebrafish embryo test methods for ED identification. The ReadEDTest is an online tool, freely accessible that does not require any user information. It is divided into 7 main sections and 13 sub-sections that contain important criteria that are requested during the validation process. A brief description of each main section and sub-section is presented in Tables 1a and 1b. The questions were inspired from the test readiness criteria proposed by Bal-Price et al. (Bal-Price et al., 2018), the template for cell-based test methods (Krebs et al., 2019) and the guidance document for describing non-guideline *in vitro* test methods (https://www.oecd-ilibrary.org/environment/guidance-document-for-describing-non-guideline-in-vitro-test-methods_9789264274730-en).

The first section 'Test Method Description' concerns the test system used in the method, the cell/zebrafish characterization, the cellular environment/fish husbandry, the exposure scheme and the technical requirements in order to assess technical readiness criteria (TRC). The following sections 'Operational readiness and data management', 'Reproducibility and Transferability', 'Historical data' and 'Standard Operating Procedures' (SOPs) investigate the reliability, the reproducibility, the transferability of the method. The data management procedure, crucial at the validation step, was built with the expertise of

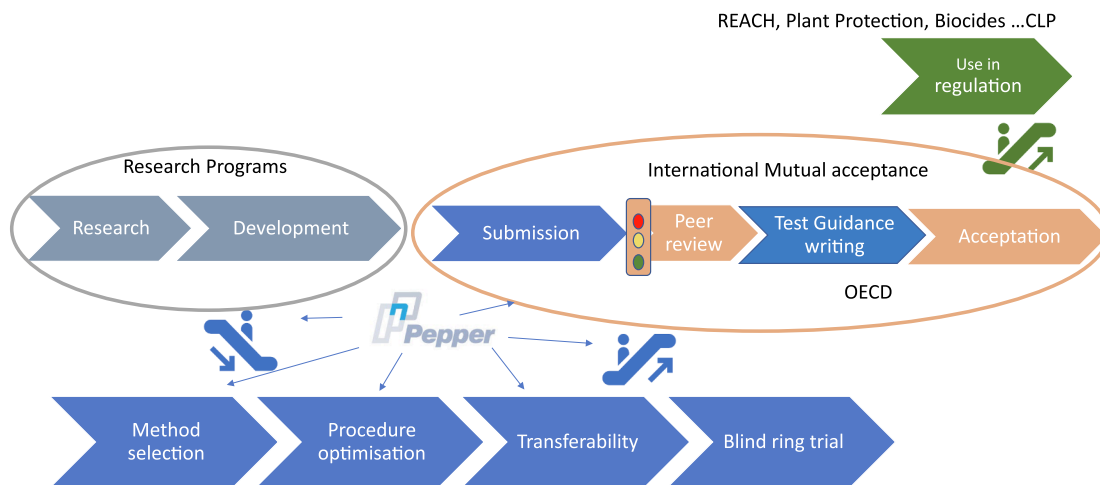


Fig. 1. From research to regulation: role of the Pepper platform in the validation process of developed test for endocrine disruptors identification. REACH: Regulation for Registration, Evaluation, Authorization and Restriction of Chemicals. CLP: Classification, Labelling and Packaging.

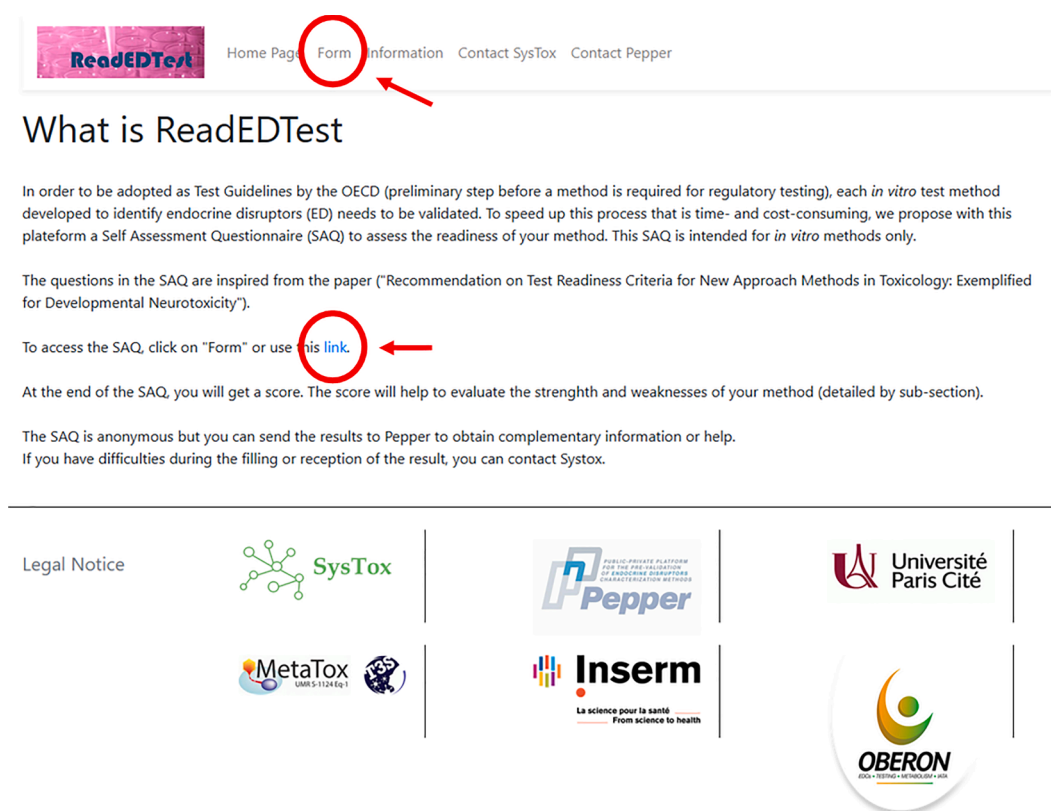


Fig. 2. The homepage of the ReadEDTest. The SAQ is accessible by clicking on the Form link.

Pepper. The section ‘Relevance of the method’ evaluates the interest of the method for regulatory purpose (i.e. is it filling a gap in identifying EDs?), and the last section ‘Laboratory resources for validation’ is related to the human and material resources already present in the laboratory.

The questions are easy to understand by researchers without any knowledge in the development of test methods or the validation process. The Fig. 3 presents some examples of the questions for the ‘cell characterization’ and for the operational readiness and data management’ sections. Since the majority of these questions concerns the information availability on the assessed criteria (yes or no), it is quite easy to answer.

2.2. Scoring calculation

The sections and sub-sections have different scores (see the scores in Tables 1a & 1b) reflecting the importance of the questions. Therefore, each section should be considered separately. The scores are attributed following the different needs for validation (e.g. the knowledge of the origin or genetic modifications of a cell line). The score limits were determined for each section and sub-section according to the importance of the questions determined by Pepper’s experts, based on their knowledge in the validation process. The Fig. 3 presents some examples of questions and associated scores. Depending on the questions, the score can be none (-), 0.5 or 1 point per question. When two questions

Table 1

Description of each section (T1a), sub-section (T1b) and their associated scores. The score limit (1b) corresponds to the value under which an alert is given.

T1a	Main section	Main section description	Score Max	
1	Test Method Description	Concerns the test system, the cell/ zebrafish characterization, the cellular environment/fish husbandry, the exposure scheme and the technical requirements in order to assess technical readiness criteria (TRC).	14	
2	Operational readiness and data management	Investigates the laboratory's ability to produce and analyze data in validation process.	3.5	
3	Reproducibility and Transferability	Investigates the availability of data on repeatability, reproducibility and the transferability of the method.	5.5	
4	Historical data	Investigate data on positive and negative controls and reference compound.	1.5	
5	Standard Operating Procedures (SOPs)	Concerns the templates or worksheets that should be written and prepared in details to allow the implementation of the method in a new (naive) lab.	3.5	
6	Relevance of the method	Investigates the regulatory relevance of the method.	1.5	
7	Laboratory resources for prevalidation	Evaluates the time and resources available and dedicated to the validation process	2	
Total Score			38	
T1b	Sub-section	Sub-section description	Score Max	Score Limit
1	Test system	Type of model, model species, justification of the model	1	—
	Cell/Zebrafish characterization	Metabolic competence, specific information on transgenic cells, information on the cell origin	9	<6.5
	Cellular environment/Fish husbandry	Culture conditions, medium supplements, particular conditions, critical components and handling steps or water quality for fish	5.5	<4
	Exposure scheme	Parameters on the culture conditions and tested compounds.	4	≤2
	Endpoint	Parameter(s) measured within the test method and technical limitations	1	—
2	Data analysis	Need for particular software, scripts or a curve fitting, benchmark threshold used	2	≤0.5
	Data management	Existing template, procedure for handling outliers and data storage	1.5	≤1
	Operational readiness	Technical requirements such as cost of consumables, workforce time and throughput of the assay	-	-
3	Reproducibility and Transferability	Availability of raw and analyzed data, number of tested compounds, number of times the method has been tested	5.5	≤2.5
4	Test method controls	Number of positive, negative controls tested	1.5	<1
5	Standard Operating Procedures	Existing templates or worksheets for the	3.5	<2

Table 1 (continued)

T1b	Sub-section	Sub-section description	Score Max	Score Limit
6	Test Method Relevance	implementation of the method, comparison with other methods Relevance of the method to identify EDs, relationship with EATS modalities	1.5	<0.5
7	Laboratory resources for pre-validation	Laboratory resources for pre-validation, lab strategy and equipment availability	2	≤1
Total score				38

are related, the point is assigned if either the answer for the first question is no or the answer for both questions is yes. The non-mandatory aspects to start a validation (e.g. the existence of a prediction model) are with no score or score limit.

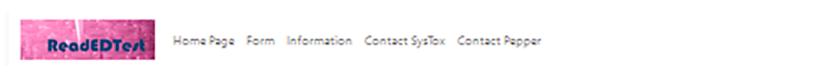
After completing the SAQ, when the score for a subsection is below the score limit, the following message “Based on this score, your method is not ready to enter validation regarding [the name of the subsection]” will appear in red. On the opposite, when the score is above the score limit, the message “Based on this score, your method is ready to enter validation regarding [the name of the subsection]” appears in green. To facilitate the visualization of the scores for all the sub-sections/sections, and the interpretation of the results from the different parts, a radar chart is automatically generated. As the score of each section is different, the scores are expressed in percentage (i.e., the obtained score/the maximal score × 100). As main result, the SAQ will give to the user an estimate of the readiness of the test according to the criteria necessary for starting the validation process.

2.3. Implementation of the SAQ

The use of the SAQ is fully anonymous. In agreement with the European Regulation on personal data ((EU) 2016/679), no personal data is collected or recorded by Université Paris Cité that developed the SAQ, and maintains the web interface. The user can choose, or not, to get in contact with the Pepper platform through a specific contact form available on the web interface. If the user wishes, he/she can share information, only with Pepper, and get help and answers about specific questions. All e-mail exchanges are managed with PHPMailer (v 6.6) (<https://github.com/PHPMailer/PHPMailer>). If the user encounters technical problems (access to the SAQ, difficulties to download the pdf with the results...), an e-mail can be sent for assistance to the laboratory of Université Paris Cité that created the web interface. The implementation of the ReadEDTest was done using the programming language PHP v7.4 (https://www.php.net/releases/7_4_0.php), which also allows a long-term maintenance. Answers and results of the SAQ can be downloaded by the user in a table format (.xls) and/or as a pdf format, both including a radar chart. All these data are generated using the programming language python (v3.9.2) (<https://www.python.org/downloads/release/python-392/>) using the modules openxl v3.0.10 (<https://openpyxl.readthedocs.io/en/stable/>), xlsxWriter 1.1.2 (<https://xlsxwriter.readthedocs.io/index.html>) and matplotlib v3.5.2 (<https://doi.org/10.5281/zenodo.7084615>) and the PHP module fpdf (v1.84) (<http://www.fpdf.org/>). In parallel, the results are sent to Pepper for information but without any personal data

2.4. Validation and evaluation of the ReadEDTest

The reliability of the ReadEDTest to provide a relevant final score was assessed using two tests already adopted as OECD TG: EASZY assay TG 250 and ERalpha Calux TG455, and four methods that are currently under development within the OBERON project: one zebrafish embryo assay and three cell-based assays.



A Test Method Description

Test Method Description concerns the test system, the cellular environment, the exposure scheme and the technical requirements in order to assess Technical Readiness Criteria (TRC).

Cell Characterization:		Scores
Is information available on:		
Sex of the donor of the cells	Yes <input type="radio"/> No <input type="radio"/>	1 / 0
Age of the donor of the cells	Yes <input type="radio"/> No <input type="radio"/>	0.5 / 0
Health status of the donor of the cells	Yes <input type="radio"/> No <input type="radio"/>	0.5 / 0
Any special pretreatments received by the cells	Yes <input type="radio"/> No <input type="radio"/>	0.5 / 0
Tissue origin of the cells	Yes <input type="radio"/> No <input type="radio"/>	1 / 0
Isolation technique of the cells	Yes <input type="radio"/> No <input type="radio"/>	-
Marker(s) for cell identity	Yes <input type="radio"/> No <input type="radio"/>	0.5 / 0
Differentiation state of the cells	Yes <input type="radio"/> No <input type="radio"/> Not Relevant <input type="radio"/>	1 / 0 / 1
Usual morphology of the cells	Yes <input type="radio"/> No <input type="radio"/>	1 / 0
Basic functions of the cells	Yes <input type="radio"/> No <input type="radio"/>	0.5 / 0

B Operational readiness and data management

This section investigates the laboratory's ability to produce and analyse data in a validation process i.e. not just once.

Data analysis		Scores
Is a particular software necessary to analyse results?	<input type="radio"/> Yes <input type="radio"/> No	-
If yes, are the software name and version identified?	<input type="radio"/> Yes <input type="radio"/> No	-
Did you create or modify a software/script/or macro to analyse results?	<input type="radio"/> Yes <input type="radio"/> No	*
If yes, has it been quality controlled?	<input type="radio"/> Yes <input type="radio"/> No	*
Is a benchmark response/threshold used?	<input type="radio"/> Yes <input type="radio"/> No	*
If yes, is there a rationale for its definition?	<input type="radio"/> Yes <input type="radio"/> No	*
Are the parameters/endpoints to be analysed well defined?	<input type="radio"/> Yes <input type="radio"/> No	0.5 / 0
Is there a need for curve fitting to estimate parameters/endpoints?	<input type="radio"/> Yes <input type="radio"/> No	*
If yes, is the model for curve fitting well defined?	<input type="radio"/> Yes <input type="radio"/> No	*

Data management		Scores
Do you have a template to report data (including experimental conditions such as day, operator...)?	<input type="radio"/> Yes <input type="radio"/> No	0.5 / 0
Is there a procedure for handling outliers: (justification for retention or exclusion)	<input type="radio"/> Yes <input type="radio"/> No	0.5 / 0
Do you have a data management scheme to store your data?	<input type="radio"/> Yes <input type="radio"/> No	0.5 / 0

* If No for the first question or Yes for two questions, 0.5 point

Fig. 3. Example of questions in the “test method description” and “Operational readiness and data management” sections and associated scores for each question.

2.4.1. The validated assays: EASZY and ERalpha CALUX

EASZY is an *in vivo* zebrafish embryo mechanism-based assay designed to detect endocrine active chemicals acting as agonist through estrogen receptors (ERs), by inducing the expression of the green fluorescent protein (GFP) driven by the *cyp19a1b* promoter (Brion et al., 2012). The EASZY assay allows the detection of estrogenic activity of chemicals on transgenic *tg(cyp19a1b:GFP)* zebrafish embryos exposed for 96 h during the embryonic stages of development. Based on EU Directive 2010/63/EU on the protection of animals used for scientific purposes, the earliest life-stages encompassed by the EASZY assay are not defined as protected and, therefore, do not fall into the regulatory

frameworks dealing with animal experimentation (Strähle et al., 2012). Hence it represents an alternative method to fish adult assay for screening estrogenic activity. The assay was submitted in November 2012 to OECD test guidelines program through a Standard Project Submission Form for its potential acceptance to the OECD working program. The information available at that time regarding the EASZY assay: basic knowledge on *cyp19a1b* in zebrafish brain (Menuet et al., 2005; Pellegrini et al., 2007), its dysregulation by (xeno)-estrogens (Cheshenko et al., 2007) and the use of the *tg(cyp19a1b:GFP)* zebrafish embryos (Tong et al., 2000) for assessing the endocrine activity of a large panel (n = 42) of chemicals (Brion et al., 2012), were used to fill

the ReadEDtest.

The ERalpha CALUX (Chemical Activated Luciferase gene eXpression) is an *in vitro* cell-based assay designed to detect hER α -mediated transactivation by measuring bioluminescence as the endpoint. It is based on the use of stably transfected U2OS cells. The validation of this test has been conducted following the performance criteria defined in the 2012 version of the TG, and the test has been included in the 2016 updated version (OECD, 2021). The test measures the activation (agonists) and also the suppression (antagonists) of the ER- dependent transcription through luciferase activity.

2.4.2. The OBERON test methods

Four test methods under development in the project OBERON (Audouze et al., 2020), one using zebrafish embryos and three using cell lines, were submitted to ReadEDTest to assess their readiness for the validation process.

The Steatogenic Assay on Zebrafish (StAZ) has been developed on zebrafish eluthero-embryos and aims to evaluate the capacity of chemicals to induce liver steatosis. This assay quantifies, after Nile red staining, the fluorescence of the liver lipid droplets following chemical exposure of zebrafish larvae between 3 and 5 days post-fertilization (dpf). Therefore, it could be considered as a NAM like the EASZY assay because at this stage of development, this *in vivo* model is outside the regulatory frameworks dealing with animal experimentation. The endpoint evaluated by this assay is the accumulation of lipid in the liver which is one major outcome of liver steatosis, the first stage of MAFLD (Metabolic-Associated Fatty Liver Diseases). This test has been tested with several molecules including EDs and allowed to identify dichlorodiphenyldichloroethene (DDE), the principal metabolite of the insecticide dichlorodiphenyltrichloroethane (DDT) as a potent inducer of liver steatosis (Le Mentec et al., 2023).

The first *in vitro* assay is a liver steatosis characterization test that determines the capacity of chemicals to induce hepatic steatosis using 2D human hepatic cell line (Eslam et al., 2020). It quantifies lipid droplet production through automated image analyzing. The assay has been tested with EDs known to be involved in MAFLD and has demonstrated to be a suitable tool for determining the direct effect of compound on lipid droplet formation and thus for the identification of potential steatotic environmental pollutants.

The second *in vitro* assay is also based on the quantification of triglycerides in cultured human hepatocytes in three dimensions. The cell line is embedded in a matrix that allows the cells to differentiate rapidly and to maintain highly specific liver function over the long-term without any addition of hormones (Rose et al., 2022). In this 3D cell model named Hepoid (Rose et al., 2021), the amount of triglycerides was assessed using the Triglyceride-Glo Assay kit from Promega (Rose et al., 2022). The principle of the measurement is to convert triglycerides into glycerol which is then measured by bioluminescence. Thus, the glycerol release from intracellular triglycerides is quantified and correlated to alteration of lipid metabolism. The 3D cells were treated during 14 days with most of the EDs of interest from OBERON project.

The last *in vitro* assay aims to determine the capacity of chemicals to alter glucose-stimulated insulin secretion (GSIS), the primary function of pancreatic β -cells (Al-Abdulla et al., 2022). Therefore, the method is able to measure an endpoint relevant to the endocrine activity of the pancreas. The assay is based on the use of a 2D human pancreatic β -cell line and allows to quantify basal insulin secretion as well as insulin release in response to stimulatory glucose concentrations. The assay has been tested with a number of paradigmatic EDs and has demonstrated to be a valuable tool for determining the direct effect of EDs on β -cell secretory capacity, and therefore for the identification of potential diabetogenic environmental pollutants.

3. Results of the SAQ

3.1. The EASZY assay and ER α CALUX transactivation assay

At the OECD level, zebrafish (*Danio rerio*) is a model fish species, but the origin of the zebrafish is seldom informed in OECD validation report and TGs. For the EASZY assay, a transgenic *tg(cyp19a1b:GFP)* model is used and essential information must be provided when using such genetically modified model regarding the stability of the transgene across generation, its rate of transmission (homozygote vs heterozygote) and the reliability of the response as measured by the reporter gene across generation with reference substances. The fish husbandry conditions need also to be specified as well as the physico-chemical properties of the test medium for an optimal use of the test. Through a series of specific question well identified in the ReadEDTest, we intended to highlight the information that “zebrafish” test developers should have regarding the zebrafish strain.

The information available when the assay was submitted in November 2012 to OECD TGs program was used to fill the ReadEDTest. As expected, the assay got an overall high score for almost all criteria (Fig. 4A), except the data management section that was beyond the limit score. This section is not essential for acceptance of a test method by the working program but must be considered carefully after approval in order to ensure the traceability of the data collected between the different laboratories and a correct reporting of the data to the validation expert group.

The ER α CALUX transactivation assay is one of the three methods included in the OECD TG No. 455 “Performance-Based Test Guideline for Stably Transfected Transactivation In Vitro Assays to Detect Estrogen Receptor Agonists and Antagonists”. The TG and referenced SOP were used to fill in the SAQ.

Some sections/sub-sections such as the cost, workforce time, the availability of a data management system in the lab or laboratory resources for pre-validation had to be estimated. Indeed, this information doesn't appear in the TG as these criteria are not directly linked to the readiness of the test. As expected, the ReadEDTest shows that the test is ready for validation for all the sections (Fig. 4B). It has to be noted that the score of “test system” and “test method relevance” sections is low because the score cannot be higher than 0.5 for a mechanistic test using a cellular model. However, such a score is sufficient and these sections are ready for validation.

3.2. The OBERON tests under development

Four European independent laboratories, partners of the OBERON project, have used the ReadEDTest tool to evaluate the readiness of their different *in vitro* tests under-development: a zebrafish larvae-based steatosis assay, two tests for the quantification of hepatic steatosis by either the estimation of lipid droplets amount or the quantification of triglycerides and a pancreatic β -cell function assay. The online tests were performed in less than 45 min, and results can be found in Table 2.

Overall, the researchers found that ReadEDTest was a very useful tool to define and get knowledge on the strengths and weaknesses of the assays in their current form. The radar plots show with easy reading the sub-sections that need to be improved (Fig. 5A-D). The final scores were 24.5 for the StAZ assay and 24, 26 and 24 for respectively, the liver steatosis characterization in 2D test, the steatosis estimation in 3D test and the pancreatic β -cell function assay.

For the StAZ assay, the results were in agreement with the available data. Indeed, the developers knew that the ‘zebrafish characterization’ and ‘fish husbandry’ sections that are not ready for validation, needed to be better characterized and checked. For the cellular tests, as expected by the researchers, there was a good score for sections usually well defined in a laboratory work such as “Test system”, “Exposure scheme”, “Endpoint”, “Test method controls” and “Test method relevance”.

The researchers also expected that the items “Data management”,

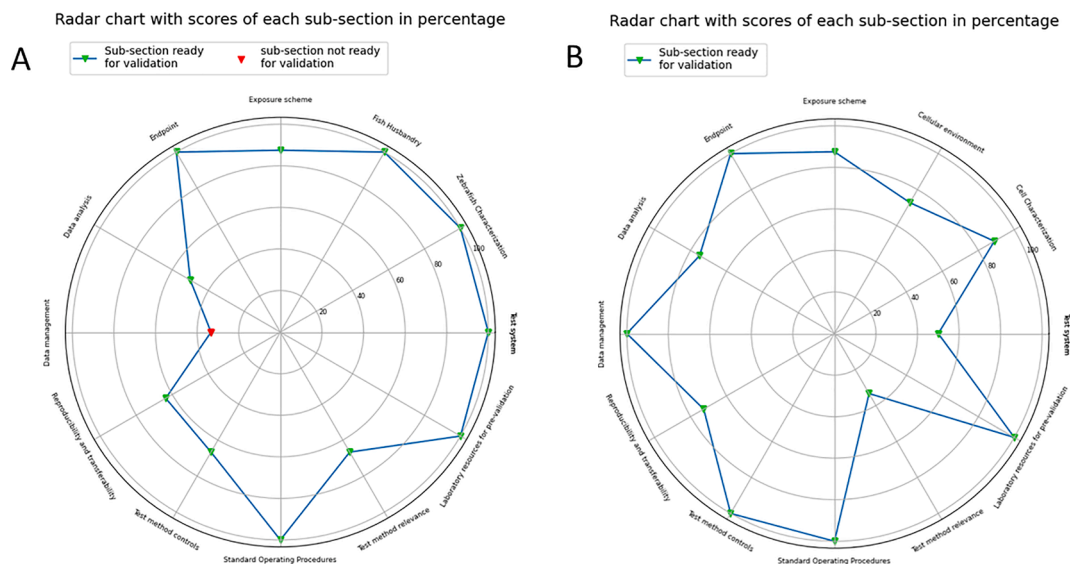


Fig. 4. Radar plot for the EASZY assay (A) and ERα CALUX transactivation assay (B).

Table 2

Overview of the results obtained with the ReadEDTest for four tests under development in the OBERON project.

Section	Subsection	Steatosis Assay on Zebrafish	liver steatosis characterization test with 2D human hepatic cell line	Steatosis estimation in human hepatocytes in 3D spheroids	pancreatic β-cell function assay	
1	Test system /1	0.5	0.5	1	1	
	Cell/Zebrafish Characterization /9	6	9	9	6.5	
	Cellular environment/Fish Husbandry /5.5	3	3.5	4.5	4	
	Exposure scheme /4	3	3	2.5	3	
	Endpoint /1	1	1	0.5	0.5	
	2	Data analysis /2	1.5	1	1.5	2
		Data management /1.5	0.5	0	0	0
	3	Reproducibility and Transferability /5.5	2.5	2	1.5	2
		Test method controls /1.5	1	1.5	1	1.5
5	Standard Operating Procedures /3.5	3	0	1.5	1	
	Test Method Relevance /1.5	1	1	1	1	
7	Laboratory resources for pre-validation /2	1.5	1.5	2	1.5	
	Total score:	24.5/38	24/38	26/38	24/38	

“reproducibility and transferability”, and “SOP” were not good. These parts are often the last sections to be completed, especially when the development of a regulatory test is not planned from the beginning, which is often the case in academic laboratories. For the StAZ assay, the situation was similar except for the SOP section that was good because the standard procedure was already prepared for the transferability and the reproducibility of the test method. After completing the SAQ, researchers realized that some key aspects should be improved or better addressed when starting the validation process. For example, the laboratory who performed the test on steatosis in 2D cell line did not expect that the cellular environment was not entirely characterized (3.5/5.5) since they had been performing and improving their test for almost two years with commercially available cells. For all of four tests, even though scores are over the limits for several items, they were not always maximal. This means that, regarding those points, the tests could go on validation process but that information/data will probably have to be completed for the final validation step. For example, even if a number of routine maintenance factors have been defined and recognized as limiting, others parameters such as the batch effects of some supplements are still not well characterized. Also, other experiments are necessary to define additional positive/negative controls; reference

compounds and historical data should also be explored as this will help to create a more robust testing procedure.

As the questionnaire is easy to understand and quick to complete, it can be completed at every step of the test development and used by several people from the same laboratory: either the person who designed the test or the persons in charge of the experiments. This has the advantage of making the whole team aware of the requirements of regulatory testing and prioritizing improvements.

4. Discussion and conclusion

One of the reasons why validation of a method takes many years is the lack of readiness of the method. The ReadEDTest online tool, presented here, aims to guide developers regarding the technical/scientific optimization of their method. It allows researchers to become aware of the lacks within the different items. In particular, the importance of historical data and of positive and negative controls, the demonstration of the stability of the test, the need for a detailed characterization of the test system, or a fixed and precise operating procedure, are examples of often retrieved missing information when tests are developed. Indeed, method developers are often unaware of the necessary requirements and

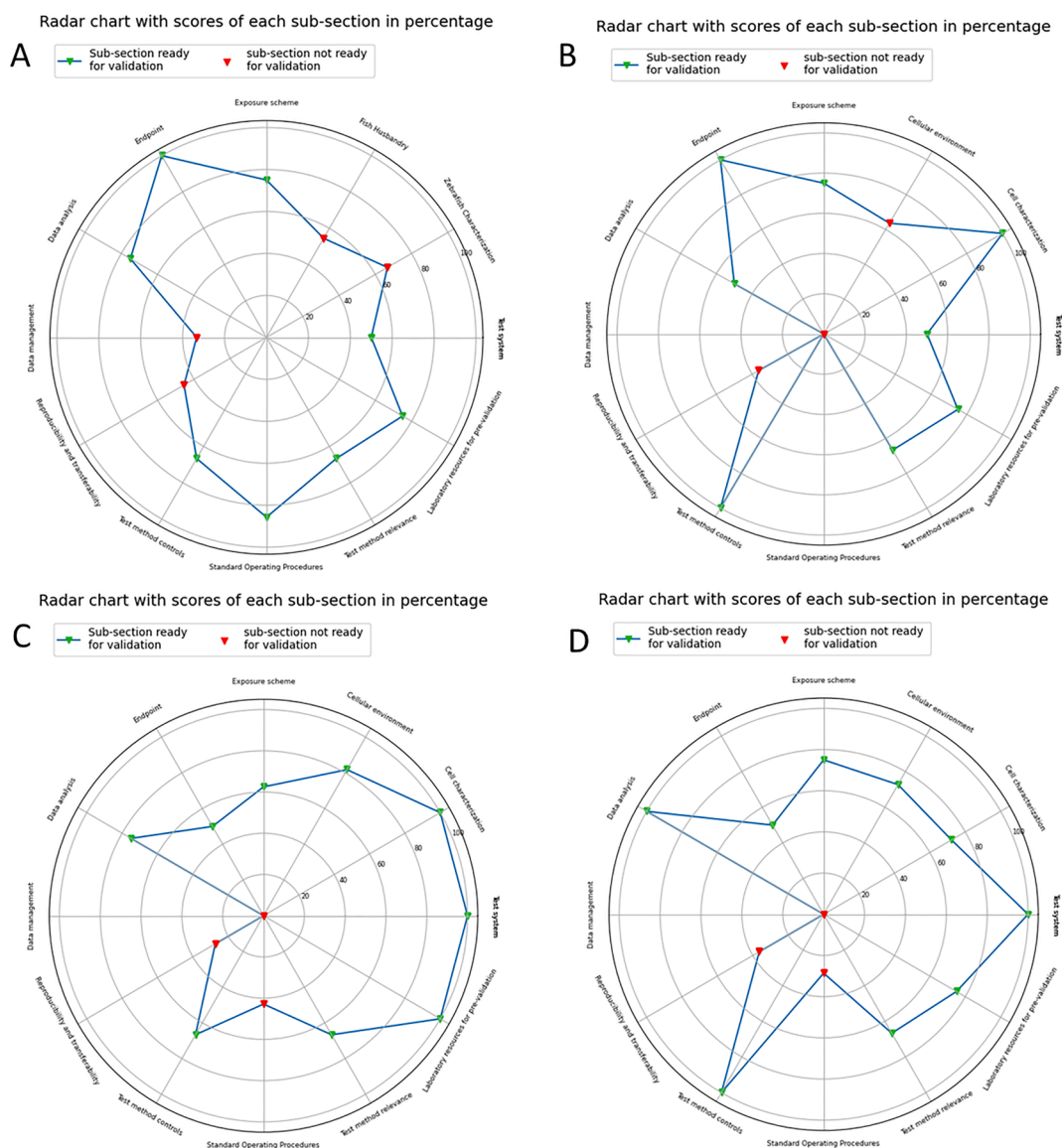


Fig. 5. Radar plots for the results from the steatosis assay on zebrafish (A), the liver steatosis characterization test with 2D human hepatic cell line (B) and the Steatosis estimation in human hepatocytes in 3D spheroids (C) and the pancreatic β -cell function assay (D).

important aspects to consider when engaging a test into a validation process. The advantage of the ReadEDTest is the compilation in one place of all these aspects informing the developers on the strengths and weaknesses of their methods.

The *in vitro* case studies demonstrated that the researchers usually have sufficient data for 9 sections that concern the characterization of the test methods. These sections have good scores (over the score limit). Even in these sections, ReadEDTest was helpful as it reveals some missing data such as historical data. However, the sections “Data management”, “reproducibility and transferability”, and “SOP” (except for the StAZ assay) present low scores as there were missing data. It is worth noticing that data management has the worst score. That justifies the generic effort of research organizations to enhance “research data management” (https://dmp.opidor.fr/template_export/2076044748.pdf). It illustrates the fact that ReadEDTest can also serve as a tool to identify what is needed for reducing the “reproducibility issues” in research (Baker, 2016). ReadEDTest is easy to use even by scientific researchers that do not have knowledge of the validation process of test.

Moreover, our tool highlights some regulatory aspects to be considered. Indeed, the goal of the Pepper platform is to foster the validation of methods for regulatory use in the assessment of chemical hazard. It is

important for developers before considering the validation of their method to keep in mind the regulatory questions that need to be addressed. Research and regulatory worlds are often too apart and developers are not aware of the regulatory needs. Furthermore, even though all the criteria are met in the ReadEDTest, it does not necessarily mean the test can be accepted since a test method submitted to OECD for its potential adoption must fill some specific needs well-identified at the OECD level. Such regulatory needs in term of test development are often identified in detailed review paper (DRP) which result from collective works on a specific theme or in European Union reports (Revised Guidance Document, 2018; European Commission, 2018). The absence of potential regulatory use of the methods will disqualify it of an OECD adoption.

Furthermore, acceptance criteria (e.g., mortality rate in control, response measured in positive/negative controls) used by OECD expert group during the review process are refined based on the validation reports from laboratories involved in the cross-validation step. Thus, the support of a pre-validation platform such as Pepper, which is in relation with OECD, can help to speed up the validation process.

The application of the SAQ on four OBERON case studies allowed the researchers to realize what is expected by validation bodies. As the

questionnaire is quick and easy (less than an hour) to complete, it has the advantage that it can be reapplied frequently, and thus reduce the risk of missing points before an official submission.

It is important to note that all the points mentioned in the questionnaire do not have the same importance, which is reflected by the various weight of each question. Developers should not wait to have the maximum score to each section before engaging into a validation. Indeed, some sections can be improved during the process and some questions may not be relevant for certain methods which would prevent to reach the maximum score. The focus should be on the threshold established for each section, highlighted by the message “Based on this score, your method is ready to enter validation regarding [the name of the subsection]”. This aspect has been highlighted by the fact that the two validated test methods do not have a maximum score for each section. Indeed, some sections such as “test method relevance” cannot be higher than 0.5 for a mechanistic test within a cellular system. However, even if the score is low, it is good enough with regard to the readiness of the method.

Altogether, the ReadEDTest intends to allow test developers which are not familiar with the requirements of an international validation, to simply and quickly have answers on several and essential aspects of their test methods so that they could easily identify strengths and limitations regarding the “maturity” of their method for potential validation.

This questionnaire ranges among new approach methodologies that allow research results to evolve into effective tools to address societal issues such as the safety of chemical substances advocated in the EU Chemicals Strategy for Sustainability: Towards a Toxic-Free Environment.

Informed Consent Statement

NA.

Data Availability Statement

NA.

CRediT authorship contribution statement

Thibault Crouzet: Methodology, Visualization. **Elise Grignard:** Methodology, Writing – review & editing. **François Brion:** Validation, Writing – review & editing. **Etienne B. Blanc:** Validation, Writing – review & editing. **Normand Podechard:** Validation. **Sophie Langouet:** Validation. **Paloma Alonso-Magdalena:** Validation, Writing – review & editing. **Philippe Hubert:** Methodology, Writing – review & editing. **Min Ji Kim:** Conceptualization, Methodology, Writing – review & editing, Project administration. **Karine Audouze:** Conceptualization, Methodology, Writing – original draft, Writing – review & editing, Project administration.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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