EUROPEAN COMMISSION
JOINT RESEARCH CENTRE
Health, Consumers and Reference Materials
Chemical Safety and Alternative Methods Unit
EU Reference Laboratory for Alternatives to Animal Testing (EURL ECVAM)

Summary Record

EU-NETVAL Meeting 10-11th October 2017, Ispra, Italy

The third meeting of the European Network of Laboratories for the Validation of Alternative Methods (EU-NETVAL) was held on 10-11th October 2017 (the agenda is included in Annex II).

Welcome and Introductory Session

EURL ECVAM welcomed all member facilities and invited experts on *in vitro* methods to the JRC. The depth of expertise within the network was acknowledged as a clear asset to the work which the Chemical Safety and Alternative Methods unit carries out. This includes the regulation of chemicals, advancing animal-free methodology for improved risk and hazard assessment whilst facilitating innovation and trade to strengthen the EU industrial base. EURL ECVAM's responsibilities were established under Directive 2010/63/EU on the protection of animals used for scientific purposes. As such, these responsibilities include: guiding research on alternative methods; coordinating validation within the EU (including co-ordination of the work of EU-NETVAL), dissemination of information on the 3Rs; facilitating stakeholder dialogue; and promoting international acceptance of alternative methods.

The current EU-NETVAL network incorporates a wide range of different technologies and has good representation across Europe. Activity levels within the network are generally good with some test facilities engaging with most activities in a highly productive way. The recent call for participation of the laboratories in the validation study of *in vitro* methods for the detection of endocrine disrupting chemicals has received a positive response from the network with twelve test facilities (TFs) covering sixteen methods. This meeting focused on the sharing of expertise not only within the network but also with *in vitro* method developers to establish confidence in the methods.

Overview of main activities and current state of the network (EU-NETVAL)

EURL ECVAM provided an update on the current activities of EU-NETVAL since the last meeting. Currently, there are 37 members of EU-NETVAL representing fifteen countries in the network. The network contributes to the development of guidance documents and training materials supporting good *in vitro* method development. Furthermore, it promotes practices that ensure scientific integrity and quality of the data generated with *in vitro* methods in order to stimulate trust by decision makers and industrial end-users. However, participation of the network members varies greatly with some members taking a very active role in the network activities and others having very little or no activity over the past year. EURL ECVAM has sent out two surveys and one call for participation in the validation study on TDs. Monitoring of the responses from EU-NETVAL members is important to ensure that the network is fulfilling its tasks (as outlined in the Terms of Reference).

EU-NETVAL has provided input in the drafting of an Organisation for Economic Co-operation and Development (OECD) technical guidance document (led by EURL ECVAM) on Good *In vitro* Method Practices for the development and implementation of *in vitro* methods for regulatory use in human safety assessment. With the EU-NETVAL activities and applying the modular approach to validation (Hartung et al., 2004), EURL ECVAM demonstrates that validation is a flexible scientific process which aims to establish the confidence that the method(s) are fit for a particular purpose. The first pilot validation project involves three selected EU-NETVAL test facilities from Sweden, UK and France for the generation of experimental data using the *in vitro* AR-CALUX method to support the development of an OECD Performance-Based Test Guideline (PBTG) and associated performance standards for Androgen Receptor Transactivation Assays (ARTA) for the detection of compounds with (anti)androgenic potential. During this validation, mechanisms were developed to ensure efficient communication and transfer of the method from the test developer to the validation partner EU-NETVAL facilities and to run the *in vitro* method under GLP conditions since the method would be part of an OECD PBTG.

In June 2017, EURL ECVAM launched a validation study within the EU-NETVAL network to assess 17 *in vitro* methods for the detection of thyroid disruptors. During the validation study, 12 EU-NETVAL test facilities will interact with the *in vitro* method developers to define the methods and establish standard operating procedures (SOPs) which can run in a Good Laboratory Practice (GLP) environment. They will also assess within-laboratory reproducibility followed by relevance assessment of the methods using a set of reference compounds. The ultimate goal is to combine several methods in a testing strategy. The 17 methods (currently 15 allocated to EU-NETVAL test fcailities) were selected based on the OECD review paper (OECD, 2014a) and with input from previous meetings and workshops. Those methods which perform well may be selected for further relevance assessment using a set of selected reference items with a view to their eventual use in a regulatory context. *In vitro* method developers and experts who have shown interest or have in-depth knowledge on *in vitro* method best scientific and quality practices are involved in this process.

The network has also contributed to information gathering exercises to support the surveillance and uptake and use of *in vitro* methods, one of the tasks of the members which are outlined in the Terms of Reference. Upon request by the OECD, a survey of EU-NETVAL was carried out to embrace advances in technologies applied to existing *in vitro* OECD TG methods, such as the OECD TG 471 on the Ames bacterial gene mutation test. The survey on the uptake by EU-NETVAL members of the throughput miniaturised Ames bacterial gene mutation test collected information to support the decisions of the OECD Expert Working Group for the development of a Detailed Review Paper (DRP). Thirty-four EU-NETVAL facilities replied to the survey including the current nine EU-NETVAL test facilities using already the miniaturised Ames. Fifty-four percent of the responders showed interest for future testing purposes while 61% showed interest in training opportunities, put into practice during the EU-NETVAL meeting. At the EU-NETVAL meeting in October 2016, a training and knowledge-sharing session on new *in vitro* skin sensitisation OECD TGs (including DPRA, h-CLAT, KeratinoSens™ and LuSens) took place. In addition, a follow-up to assess the impact of the training in

terms of practical implementation in more EU-NETVAL facilities has been assessed by a dedicated survey which was also discussed at the meeting.

Updates on AR-CALUX *in vitro* method

EURL ECVAM is coordinating the validation study of an *in vitro* method that can determine androgenic and anti-androgenic activity of compounds: the AR-CALUX method developed by BDS (Netherlands). This study represents the first activity undertaken with 3 labs of EU NETVAL (CitoxLAB, Envigo, Rise). The progress of the ongoing validation study was presented and projection of conclusion of the experimental part end 2017/early 2018 on the basis of the results obtained by all 3 labs. The tasks ahead to achieve an OECD Performance Based Test Guideline (PBTG) accompanied by Performance Standards (PS) were presented: the drafting of the validation report, the drafting of the texts for PBTG and TG, followed by reviews by ESAC and OECD (VMG-NA, WNT). Detail was provided on the development and application of the classifier in the AR-CALUX method, to be used to determine if test chemicals show (anti-) androgenic properties. This classifier includes a Specificity Control for the antagonist part of the method to identify false positive chemicals. Some results from Study 1 and Study 2 of the AR-CALUX validation study were presented.

Each of the participating test facilities gave feedback on the study. Common challenges include the demand on resources, selection of appropriate consumables and reagents and time for conducting the validation study. The differences in funding between the Member States were also apparent from the feedback.

EU-NETVAL Survey on uptake of OECD Test Guideline skin sensitisation *in vitro* methods

EU-NETVAL is important for knowledge sharing, but also for the surveillance of uptake and use of validated *in vitro* methods. Indeed, this is one of the tasks of the network members outlined in the Terms of Reference. In order to assess the impact of this knowledge sharing within the network and as a follow-up to the training which was provided at the 2016 meeting, EURL ECVAM launched a survey of EU-NETVAL members on the uptake of OECD Test Guideline (TG) skin sensitisation *in vitro* methods. Nineteen of the thirty-seven test facilities had responded to the survey prior to the meeting. The preliminary results were discussed during the meeting and seven test facilities (Abich, Charles River Laboratories Edinburgh, CiToxLAB, Envigo, Eurofins and Vitroscreen) had the opportunity to present their practical experience with the h-CLAT, DPRA and Keratinosens methods (see presentations). This was much appreciated by the participants and there was expression of a marked interest for a continued discussion on this topic and a forum for providing feedback to OECD test guidelines. EURL ECVAM will investigate the possibilities on how to facilitate this.

The survey was reopened following the meeting as all EU-NETVAL members are required to reply.

Updates on Directive 2010/63/EU

Directorate General for Environment (DG ENV) presented the follow up to the European Citizens' Initiative (ECI), Stop Vivisection, specifically on Action 4 which was the European Commission's (EC) commitment to facilitate a scientific dialogue on the validity of the animal model. This dialogue took place at the EC conference, Non-Animal Approaches - The Way Forward, which was held in Brussels on 6-7th December 2016, gathering scientists and other stakeholders across the EU to discuss the possibilities and challenges on moving towards non-animal approaches. The Conference report is available and all involved are encouraged to look and take up recommendations where appropriate.

Article 58 of Directive 2010/63/EU on the protection of animals used for scientific purposes requires the Commission to carry out a review on the Directive by 10th November 2017. The process was explained and the problems in relation to the early timing of the review deadline were underlined, which include, inter alia, on-going conformity checks on national legislations, lack of data on the implementation (due at the end of 2018), lack of EU statistics on animal use (due November 2019) and accommodation and care standards being applicable only since the beginning of 2017. As a result users, Members States and other stakeholders have only limited experience on the functioning of the Directive. In reference to new structures designed to accelerate the development, validation and uptake of new alternative methods, EU-NETVAL plays a key role. The Review report and the accompanying Staff Working Document will be published on the Commission web-site. DG ENV invited participants to examine especially the Staff Working Document that contains a number of recommendations for different stakeholders to take up, as appropriate, with the common aim of improving the attainment of Directive objectives.

Discussions following the presentation focused on the funding of activities to develop alternative *in vitro* methods. Member States are aware of the importance of funding and there are reports on the DG ENV website which illustrate what some Member States are doing to facilitate the development, validation and promotion of alternative approaches at the national level. However, the authorities responsible for funding for research and validation activities vary greatly between Member States and it is therefore important to understand who is/are the potential funding body/ies available in the respective Member State for assay development. The National Contact Points for the implementation of Directive 2010/63/EU are available on DG ENV website. DG ENV agreed to investigate with EURL ECVAM in which way funding requests could be supported, such as a letter of support to be presented to Member State authorities/other funding bodies demonstrating the need for relevant funding.

EU-NETVAL survey on miniaturised Ames *in vitro* method(s)

As a contribution to the OECD Expert Group Project on the comprehensive review of the miniaturised versions of the Ames bacterial gene mutation test, an exploratory survey was extended to EU-NETVAL. The involvement of EU-NETVAL was sought to support the surveillance of the uptake and use of validated *in vitro* methods, which is one of the tasks of the network. The survey was launched on May 12, with the deadline on June 9, 2017. Thirty-four EU-NETVAL facilities replied to the survey including the current nine EU-NETVAL test facilities using already the miniaturised Ames. Fifty-four percent of the responders showed interest for future testing purposes while 61% showed interest in training opportunities. For this reason, the results of the survey have been shared with the members and two separate training sessions on miniaturised versions of the Ames test were put into practice. Some of the EU-NETVAL member laboratories presented their test methods to the other participants. Step-by-step methodologies were shown, with practical demonstrations and provision of materials. The sessions saw the participation of the interested EU-NETVAL members with time for questions, observations, and fruitful discussion.

In vitro methods for the detection of thyroid disruptors

EU activities on thyroid disruptors

EURL ECVAM described the background to the concern for substances with endocrine disrupting properties and particularly those substances disrupting thyroid hormone action which may lead to many types of adverse effects, such as brain development. The EFSA opinion of 2013 indicated the lack of standardised *in vitro* mechanistic assays for substances affecting the thyroid hormonal axis, as well as the OECD 2014 call to OECD countries to support the development of such assays as described in the OECD scoping document 207.

The outcome of two projects contracted by DG ENV over 2016 - 2017 which led to a workshop in March 2017 and another in May 2017 was also presented. The first focused on ways to improve current approaches to the identification of thyroid disrupting substances, whereas the second related to setting priorities for the further development and validation of test methods and approaches to evaluate endocrine disruptors in general.

The outcome of both workshops reconfirmed neurodevelopmental effects caused by thyroid hormone disruption as one of the highest priorities for method development as well as the need for *in vitro* assays to cover the different modes of action for thyroid hormone disruption.

Updates from OECD and Importance of thyroid activities - Organisation for Economic Cooperation and Development (OECD)

An update on the status and development of OECD projects related to the thyroid pathway was provided. The conceptual framework for endocrine testing which includes five levels, ranging from existing and non-test data, *in vitro* mechanistic assays, *in vivo* mechanistic assays, and *in vivo* assays that provide comprehensive data was reviewed. At each level, available OECD test guidelines that include thyroid-relevant endpoints were briefly discussed. These guidelines include a variety of key events and life stages, but currently, there are no OECD Test Guidelines that provide mechanistic data. In order to bridge this recognised data gap, OECD published a 2014 Guidance Document (No. 207) evaluating the state of readiness and toxicological relevance of *in vitro* assays for the thyroid pathway. The same year, the OECD Advisory Group for Endocrine Disrupter Testing and Assessment requested proposals for *in vitro* thyroid assays and received no responses. Internationally harmonised *in vitro* thyroid test methods are a critical to anchor adverse responses observed in *in vivo* animal studies to a thyroid mechanism of action.

Outcome of the call for EU-NETVAL participation in the validation study of *in vitro* methods for the detection of thyroid disruptors

EURL ECVAM detailed the call to EU-NETVAL members for participation in the validation study of *in vitro* methods for the detection of thyroid. EURL ECVAM collected information on 17 methods, taking primarily into account the information reported in the OECD review but also the OECD Detailed Review Paper (2006), and feedback received at various meetings (e.g., the EU NETVAL meeting of 2016, OECD Validation Management Group-Non-Animal meeting 2016 and the DG ENV/ANSES Thyroid Disruptor workshop in 2017 (DG ENV, 2017)). Further information has been retrieved for each of these *in vitro* methods, and on the basis of this information, a set of *in vitro* methods, covering all of the eight blocks identified as known targets of thyroid disruption (except epigenetic changes) and described in the OECD review (OECD, 2014a), have been selected for the EURL ECVAM coordinated validation study.

Validation study of *in vitro* methods for the detection of thyroid disruptors

This validation study will consist of two parts: part one will define the methods and assess their transferability and reliability, and part two will assess the overall relevance based on the underlying mechanisms of the selected *in vitro* methods using the same set of reference chemicals (test items) for all test methods. During the meeting, some of the involved method developers and corresponding EU-NETVAL test facilities were introduced to each other and had the opportunity for face to face knowledge exchange.

Review of the *in vitro* methods

EURL ECVAM presented an overview of the 17 selected methods included in the EU-NETVAL call. These methods are suitable to capture different mechanisms of thyroid disruption, according to the description provided in the OECD Scoping document published in 2014 and related publications. In particular, for each method the name and the affiliation of the original test method and, when needed, the test system developers were indicated, together with the name of the EU-NETVAL institution that was assigned to each method.

Methods 2b, 2c, 2d, 3a, 3b, 4a, 6b, 7a and 8a were presented by method developers who were present at the meeting (see agenda in Annex II).

Consideration of critical aspects in relation to *in vitro* method definition

From an outline protocol to an SOP

An outline protocol for a given method can be designed based on information provided in the literature and the documents provided by the test method developer (when available). As an example, method 3a (the ANSA protein binding assay) was presented, together with some preliminary data proving the transferability of the method.

A short summary was given with regards to the work done in the EURL-ECVAM laboratory regarding method 3a. It was shown that the IC_{50} values obtained with the "standard" control items T3 and T4 were comparable with those in the literature. It was also demonstrated that the method was robust in terms of incubation time and storage of the control item T4. Even though not all data with regards to test items could be presented due to the limitation of the time available, it was shown that the method is able to detect the IC_{50} values of various types of suspected thyroid disrupting chemicals such as polychlorinated biphenyls (PCB's), materials used in plastics (like Bisphenol A), flame retardants (polybrominated diphenyl ethers) and disinfectants used in soaps. When available, the found IC_{50} values were comparable with those in the literature.

Test system management

Cell and tissue cultures are important tools in regulatory testing for adverse effects of compounds of various kinds. Unfortunately, many cell lines are misidentified or contaminated with other cells, wasting substantial time, effort and laboratory resources and potentially invalidating published data or study reports. To standardise cell line authentication, standards have been developed and the draft OECD GIVIMP guidance document elaborates on this topic. The importance of correct test system management was emphasised as there are numerous examples of cell line cross contamination and misidentification. Good cell banking practice and cell line authentication will be applied for the thyroid validation study, with support from EURL ECVAM as was done for the AR-CALUX validation study which was again illustrated.

The art of cell authentication

Dr Glyn Stacey, National Institute for Biological Standards and Control (NIBSC), UK

There is a history of issues associated with cell line contamination which highlight the importance of cell authentication. Current techniques for controlling for cell identity were also indicated as well as criteria to consider when sourcing new cell lines. The GCCP principles established by Coecke *et al* in 2005 were reviewed and it was described how these remain valid today but are undergoing a process of updating in a t4[1] workshop series. Workshop 1 had already been published (Pamies et al., 2016) and workshop 2 is in press. A writing group is now drafting a GCCP 2.0 document which would

[1] t4 is the Transatlantic Think Tank of Toxicology

include a new section to deal with microphysiological culture systems, 3D culture and human stem cell lines.

Fetal Bovine Serum (FBS): Past – Present – Future

Jan van der Valk, 3Rs-Centre Utrecht Life Sciences, Utrecht University, Utrecht, Netherlands

Foetal Bovine Serum (FBS) is still being used as the universal medium supplement to grow and maintain cells and tissues. Its use in cell and tissue culture presents five significant issues:

(i) the degree of suffering experienced by the unborn calf during blood collection for the production of FBS;
(ii) inappropriate cellular growth profiles and physiological responses of cells in media containing FBS;
(iii) the large variability of FBS such that it is very difficult to even ensure consistent and well controlled *in vitro* cell culture between batches;
(iv) the fraud-problem;
(v) demand exceeding supply issues.

Recent years showed tremendous efforts in the establishment of human platelet lysates as one of the valuable alternatives to FBS as cell culture supplement. In addition, for several applications, chemically-defined media have become available. The recently established serum-free database (fcs-free.org) facilitates the identification of existing serum-free media.

Strategies for the development of a chemically-defined growth medium are available, as well as for cell adaptation procedures.

For further details, see the workshop report: Fetal Bovine Serum (FBS): Past – Present – Future

Test item management

EURL ECVAM presented an overview of test item (chemicals) management at EURL-ECVAM. Chemicals are required for internal use and distribution to external partners, normally acquired as coherent sets relevant to particular projects. Inventories of quantities, supply, cost, ordering, etc. are compiled (*Excel* spreadsheets) together with material safety data sheets (MSDS, indicating handling precautions and storage conditions) and certificates of analysis (CoA) for quality assurance, lot number, and expiry date (if available).

Significant support has been provided during 2017 to the EUToxRisk project, a multinational programme integrating methods for repeat dose systemic and reproduction toxicity. In particular, chemicals have been managed for aliquot distribution to case studies as follows:

- liver toxicity of phenols (22 chemicals, 7 labs)
- mitochondrial toxicity of pesticides (23 chemicals, 6 labs)
- liver & kidney toxicity of phenoxy-carboxylic acid herbicides (24 chemicals, 4 labs)
- lung & neurotoxicity of di-ketones (volatile!) (8 chemicals, 2 labs).
- cross-systems testing (19 chemicals, 11 labs).

The aim of the workshop was described and this is to look at nephelometry compared to visual inspection for the assessment of solubility. Solubility is relevant to chemical preparation for *in vitro* assays, ensuring compatible concentrations are used, avoiding precipitation issues with consequent errors in dose-response analyses. Solubility determination confirms initial stock solution

concentrations in solvent, with monitoring of subsequent stability on dilution and incubation in assay medium.

Performance of the *in vitro* method

Following the OECD second round of commenting on the Good *In Vitro* Method Practices (GIVIMP) guidance document, it was agreed that Chapter 08, on Performance of the method, should be revised so as to place the emphasis on checking performance of *in vitro* methods developed in-house and separated from OECD concepts on PBTG/PS and ring-trial validation, as these are already addressed in the OECD Guidance Document 34.

The focus of Chapter 08 should address analytical method validation parameters as defined by FDA and ICH Q2R1, which are to be used as a starting point, as many of these parameters might not be relevant or practical to consider for all *in vitro* methods. The application of existing guidelines and guidance documents (e.g. EMA 2011, FDA 2001, ICH 2005, IUPAC 2002, etc.) was addressed. An exchange of information on and experience with the process of in-house validation with *in vitro* methods developers will be used to feed into the revision of Chapter 08 of GIVIMP.

Follow-up discussions on topics raised by partners

Meeting participants, in particular the method developers, commented on how important this sharing of information is and that the meeting provided an excellent platform to obtain interesting and helpful feedback and questions. The knowledge sharing sessions were widely appreciated and the necessity to establish a forum to facilitate knowledge exchange was discussed. A dedicated space in which all test facilities could share their comments on OECD TGs and to summarise these to present to the OECD would be considered very useful.

Follow-up actions:

- EURL ECVAM will investigate setting up a forum for knowledge exchange (possible with CIRCABC or another platform)
- All meeting participants will be given access to the EU-NETVAL interest group on CIRCABC
- EURL ECVAM will bring the necessary method developers of a thyroid method that were not at the meeting in contact with their EU-NETVAL partner test facility.
- DG ENV and EURL ECVAM will explore how funding requests to MS authorities and other funding bodies could be supported (such as a letter of support).
- Glynn Stacey will provide support to EURL ECVAM to identify the correct procedure for characterisation and authentication of the cellular test systems used in the thyroid method. Method developers will be asked to support this activity.
- Jan van der Valk will provide support to EURL ECVAM to identify alternative FBS-free chemically based media for the test systems to be used in the thyroid validation study. Method developers will be asked to support this activity.

Annex I

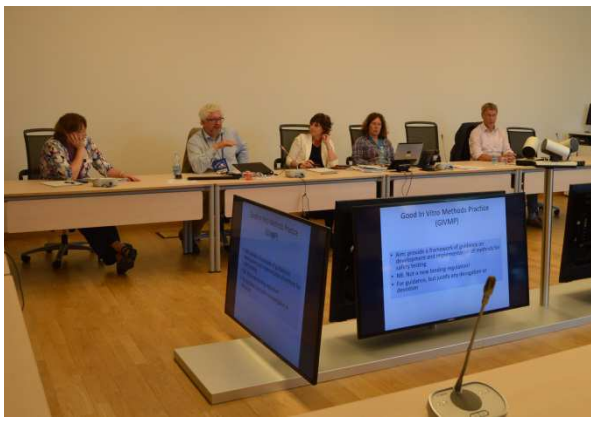
Interactive Knowledge Sharing Sessions

Some of the discussions which took place during the interactive knowledge sharing sessions are summarised below.

In vitro methods for the detection of thyroid disruptors with expert advice from method developers

Face to face knowledge exchange was facilitated between the *in vitro* method developers and the participating test facilities. It was noted that not all of the *in vitro* method developers were present at the meeting. However, further interactions between the method developers and the participating facilities can be organised following the meeting.

EURL ECVAM will provide statistical support to the study. During the first year, interactions between the method developers and the participating laboratories will take place to ensure that the proper statistical support is in place.



GIVIMP - Interactive session with *in vitro* methods developers and session participants on in-house validation of *in vitro* methods

The goals of the session were to exchange information and experience, to discuss the applicability of existing guidance documents and to provide recommendations on assessing the performance of in-house developed *in vitro* methods for GIVIMP. Method developers' backgrounds ranged from academia to those working in a regulated environment under a formal quality system. In general those who applied a formal quality system (e.g. ISO 17025, GLP) performed in-house method validation, often applying existing guidance documents or guideline, while those who did not work in a quality system had a less formal approach to in-house validation.

The importance of terminology was discussed as validation means different things to different people, e.g. those from research had a different understanding of validation than those who working in the regulatory field. The validation parameters (e.g. specificity, sensitivity, etc.) may also be interpreted differently depending on the context (e.g. sensitivity as assessed in-house is a different concept than sensitivity when assessed in a ring-trial validation).

In academia the term validation is perceived as something very official and possibly a very rigid process, however most developers explained they do look at aspects like reproducibility, criteria for

assay acceptance (e.g. the response of the positive control, linearity). The criteria however that were used were more implicit rather than derived in a clear and consistent way.

The importance of having clear definitions in GIVIMP was stressed to avoid confusion. It was agreed that not all parameters described in GIVIMP would relevant for all assays, however, a measure of reproducibility of the assay, together with some criteria when to accept or reject an assay results should always be in place.

Miniaturised Ames *in vitro* method

In an effort to compare the Ames agar plate test and the Ames Microfluctuation Assay (Ames MPF) and identify the advantages and potential pitfalls of the latter, the two assays were briefly described.

The practical advantages of the miniaturised 384-well plate Ames test were shown performing a demo in the laboratory. The miniaturised Ames is faster, requires less plastic ware and sample (including material of animal origin) and is able to reveal cytotoxicity. Moreover, data from peer-reviewed one-to-one comparisons were presented showing that the Xenometrix miniaturised Ames test is more sensitive than the pre-incubation Ames test for weakly positive/equivocal test items.

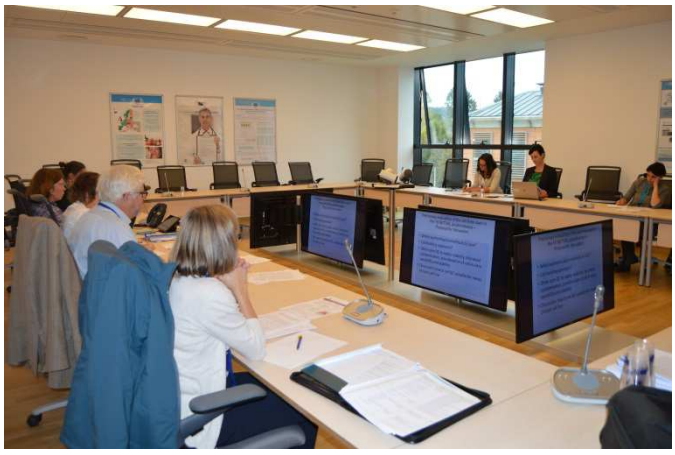
Results are currently being collected using the miniaturised 384-well plate Ames MPF test for an exhaustive comparison with the agar plate Ames test in anticipation of a potential new OECD guideline for the miniaturised test.

Test system management

Good Cell Culture Practice (GCCP) and cell authentication

It was emphasised that quality assurance is important for test systems management, besides quality control. It is necessary to take adequate measures to avoid problems.

Use GCCP (Coecke et al. 2005), which is based on the following principles:



1) Understand the system you are working with

2) Assure quality of materials, methods and procedures (Virus infections can hijack the cell to function properly.)

3) Documentation

4) Safety for individuals and environment (e.g. containment, physical and chemical)

5) Compliance with laws and ethical principles (GMO, pluripotent stem cells (database www.hpscreg.eu showing that the cells were ethically sourced))

6) Education and training

GCCP 1.0 remains valid, but must be updated with new techniques etc. EU-NETVAL members can still comment on the draft 2.0. GIVIMP is considered to be best practice, it was emphasised that it is not a new regulation but when you deviate from it, you need a good reason and must justify this.

Confluency is very subjective and is rarely correctly determined.

For the thyroid project, there needs to be a scientific approach for each test system to decide on the authentication method, the cell banking approach, how to manage primary cells, the type of quality control to apply (viability, contamination), etc.

Recommended QC requirements for EU-NETVAL:

- Carefully select your cell lines
- Screen all new cells for mycoplasma (ideally two methods)
- Establish a master cell bank and WCB. Challenge: how to do the banking of primary cells or ensure they are constantly available.
- Make a checklist before brining your cells into the laboratory on what you will check in the test system.
- Cell bank tests: Mycoplasma (two), select tests for viability, authenticity, stability based on the nature of the cell line.
- Other tests that add value for functionality.

Check for all test systems the identity, viability and critical attributes.

Assays are needed to check primary cells. Pre-use qualification of cells is maybe done by some people already. Cell lines are not homogeneous usually (STR profiling might not pick that up).

Collaboration between the EU-NETVAL facility and the developers is needed to propose for each test system what must be done to test gene expression, functionality etc. EURL ECVAM must do a patent search also. In the initial stage we need to select aspects that should be checked. As a minimum the nature of the cells must be known and what to check in the cell type.

It was advised that central cell banks should be established where possible and distributed from there. The supply of cells should be guaranteed, so maybe a cell bank must be found for banking and characterisation. Reference strains that come from different centres are not necessarily the same.

Where possible, the test system should be of human nature.

All types of test systems have an infection risk. Checking the source of the cells takes some time and requires some work. Splashes and aerosols can transmit mycoplasma (but there must be a source). HeLa Cells are also a huge contaminant.

<u>Serum free media and Serum alternatives</u>

Disadvantages of FCS: fraud cases showed that some producers of serum diluted it. Prices are high. Serum is used for many things, even for the production of artificial meat. It is expected that in the next 2-5 years the demand will exceed the supply. Quality of serum is not always good and you don't know what is in there.

In 2008 there was already an ESAC statement pushing for the use of Serum alternatives.

Several types of serum free media exist:

- Animal/human tissue or plant extracts (undefined).
- Protein free media, animal-derived component free media (all undefined).
- chemically defined medium (defined, but most difficult).

Protein free media e.g. Charcoal stripped bovine serum, has greater batch to batch variability.

Chemically defined medium must be developed for each cell type. Common factors between all existing chemically defined medium must still be identified. Start with basal medium DMEM and Ham's nutrient mixture F12, 50:50. Supplements are: hormones, growth factors, protease inhibitors.

In the absence of chemically defined medium, platelet lysates are a good alternative. These address only the ethical issue. They have growth factors, are safe, are clinically tested and are of high quality. They are human based and there are fewer differences between batches. For this purpose expired blood is used, which is not good for clinical use, but can be used for this purpose. They contain growth factors and are still undefined. The best way to go is to create chemically defined media.

To test if serum free medium is okay, you must check pre-defined performance standards. The FBS contains many growth factors, much more than in the *in vivo* situation. The population doubling time and level, attachment, phenotypic stability, genetic stability, cell fragility and size are performance standards that must be monitored when changing to chemically defined medium.

Zhao et al. 2017 propose factorial design and real-time cellular analysis to check cell growth and viability, using image analysis and factorial design.

3D models are much less dependent on FBS, their own growth factors feed neighbouring cells.

The FCS free Database FCS-free.org is established with help of UK. There are, besides others, also commercial serum free media in the database that work well, but components are not known. For CHO cells 4 media are available in the database. When using the commenting function, other scientists can be helped to improve quicker the media used for cells.

The facility should first look into this database if something is available. For all test systems used in the thyroid project, either in literature or the database there are alternative media available. GIVIMP also addresses serum free media. The method developers can work together with the EU-NETVAL facility to explore the feasibility of changing to serum free medium.

Action: method developers will be asked to assist with the test system characterisation and serum free media.

Nofer Institute of Occupational Medicine presented their test system management:

Source of cell lines: ATCC, ECACC, DSMZ, JRCB cell bank. They register the cells, culture immediately and determine cell line characteristics. There is a list with cell lines used for GLP. Freezing and thawing is managed in GLP compliance by recording freezing and thawing.

Quality control performed:

- doubling time,
- mycoplasma

- Viability after thawing
- Viability for the test (at least 95%)
- Endpoint value for negative and positive

Cell lines are stored in a -150 freezer.

It was advised that the warming due to the opening of the freezer in the top can be too high. Temperature shifts during operations may have serious effects on the cells. Likewise, the opening of the incubator door has biological effects. Other participants explained briefly what they do to characterise their cells: for cells which are stored at -160 °C, the master bank will remain of good quality and no refreshment is needed. At -150 °C, it depends on the freezing medium. It was suggested to check viability every 5 or 10 years.

It is important to take use and quality control into account for cell banking in order to make sufficiently large banks.

Test item management

In two sub-group sessions, an overview of solubility testing at EURL-ECVAM was presented, comparing nephelometry and visual inspection approaches.

Solubility is relevant to chemical preparation for *in vitro* assays, ensuring compatible concentrations are used, avoiding precipitation issues with consequent errors in dose-response analyses. Solubility determination confirms initial stock solution concentrations in solvent, with monitoring of subsequent stability on dilution and incubation in assay medium.

The two sub-groups visited the laboratory where criteria adopted for algorithmic solubility evaluation by nephelometry were explained, including a practical demonstration of solubility measurement using the instrument.

During the feedback session, it was found that none of the participating facilities actually have nephelometers.

References

Coecke, Sandra, Michael Balls, Gerard Bowe, John Davis, Gerhard Gstraunthaler, Thomas Hartung, Robert Hay, et al. 2005. “Guidance on Good Cell Culture Practice. a Report of the Second ECVAM Task Force on Good Cell Culture Practice.” *Alternatives to Laboratory Animals: ATLA* 33 (3): 261–87.

Zhao, Ai, Fahai Chen, Chunhong Ning, Haiming Wu, Huanfang Song, Yanqing Wu, Rong Chen, et al. 2017. “Use of Real-Time Cellular Analysis and Plackett-Burman Design to Develop the Serum-Free Media for PC-3 Prostate Cancer Cells.” Edited by Suresh kumar Subbiah. *PLOS ONE* 12 (9): e0185470. doi:10.1371/journal.pone.0185470.

Annex II - Agenda

Meeting of EU-NETVAL member test facilities

1st day: 10th October 2017
Building 58, Auditorium

09:00-10:00	**Welcome and introductory session** Overview of main activities (**including Good *In Vitro* Method Practices (GIVIMP)** of EU-NETVAL – *Elisabet Berggren and Sandra Coecke, EURL ECVAM* Updates on current state of the network and operational information – *Tracey Holley, EURL ECVAM*
10:00-11:00	**Updates on AR-CALUX *in vitro* method** Validation of the Transcriptional activation assay for detection of androgenic activity of chemicals (AR-CALUX) *in vitro* method – *Anne Milcamps and Roman Liska, EURL ECVAM* ***Feedback from participating EU-NETVAL test facilities – CiToxLAB; Envigo; RISE*** ***Feedback from test developer– Harrie Besselink, BDS***
11:00-11:30	Coffee break
11:30-12:00	**Updates on Directive 2010/63/EU– *Susanna Louhimies, DG Environment***
12:00-12:30	**EU-NETVAL Survey on uptake of OECD Test Guideline skin sensitisation *in vitro* methods** – *Tracey Holley, EURL ECVAM* ***Flash presentations by EU- NETVAL members on use and related issues of OECD TG skin sensitisation in vitro methods in their facilities – ABICH; Charles River; CiToxLAB; Envigo; Eurofins Biopharma Product Testing; National Institute of Public Health; Vitroscreen***
12:30-13:30	Buffet lunch
13:30-14:30	**EU-NETVAL survey on miniaturised Ames *in vitro* method(s)**– *Federica Madia, EURL ECVAM* **Experiences of running the miniaturised Ames *in vitro* methods – *Flash presentations from EU-NETVAL – Accelera; CiToxLAB; Izsler; VITO NV***
14:30-15:30	***In vitro* methods for the detection of thyroid disruptors** EU activities on thyroid disruptors – *Sharon Munn and Elise Grignard, EURL ECVAM* **Updates from OECD and Importance of thyroid activities –** ***Patience Browne, OECD***
15:30-16:00	Coffee break
16:00-17:20	**Outcome of the call for EU-NETVAL participation in the validation study of *in vitro* methods for the detection of thyroid disruptors** Overview of EU-NETVAL response - *Sandra Coecke, EURL ECVAM* Introduction to test developers: ***Toine Bovee, (2b); Alexius Freyberger[1], (2c); Antonio De la Vieja[2], (2d); Timo Hamers, (3b); Kostja Renko, (4a); Harrie Besselink, (6b); Demetrio Raldua, (7a); Arno Gutleb, (3a, 8a)***
17:30	Departure
19:30	Dinner

1 Not present during the meeting

2 Test system provider

JRC Ispra, 10th – 11th October 2017

2nd day: 11th October 2017
Building 58, Auditorium

09:00-10:00	**Validation study of *in vitro* methods for the detection of thyroid disruptors (cont.)** – *Sandra Coecke, EURL ECVAM* Review of the *in vitro* methods – *Francesca Pistollato, EURL ECVAM with* ***Toine Bovee, (2b); Alexius Freyberger[1], (2c); Antonio De la Vieja[2], (2d); Timo Hamers, (3b); Kostja Renko, (4a); Harrie Besselink, (6b); Demetrio Raldua, (7a); Arno Gutleb, (3a, 8a)***
10:00-11:00	**Consideration of critical aspects in relation to *in vitro* method definition** • From an outline protocol to an SOP - *Francesca Pistollato and Jan De Lange, EURL ECVAM* • Test system management – *Lotta Bostroem and Sandra Coecke, EURL ECVAM* **The art of cell authentication – *Glyn Stacey, NIBSC*** **Fetal Bovine Serum (FBS): Past – Present – Future –*Jan van der Valk, 3R Centre Utrecht Life Sciences*** • Test item management – *Tom Cole, EURL ECVAM* • Performance of the *in vitro* method – *Gerard Bowe, EURL ECVAM*
11:00-11:30	Coffee break
11:30-13:00	**Specific interactive knowledge sharing sessions (in building 101 and EURL ECVAM laboratories where applicable)***
13:00-14:00	Buffet lunch (Building 58, Auditorium)
14:00-15:30	**Specific interactive knowledge sharing sessions (in building 101 and EURL ECVAM laboratories where applicable)***
15:30-16:00	Coffee break (Building 58, Auditorium)
16:00-17:20	Wrap up session and **additional items raised by EU-NETVAL members**
17:30	Departure

Annex II – Agenda Continued: Specific interactive knowledge sharing session

2nd day: 11th October 2017 continued

*** Specific interactive knowledge sharing sessions** will be allocated based on expressed preferences and include:

1.i *In vitro* methods for the detection of thyroid disruptors with expert advice from test developers

An interactive session between EURL ECVAM, the *in vitro* method developers and the EU-NETVAL test facilities that responded positively to the call for the validation of *in vitro* methods for the detection of thyroid disruptors. **(Morning session only)**

Facilitators: Francesca Pistollato, Elise Grignard, Marlies Halder, Alfonso Lostia, Anne Milcamps, Sharon Munn and Camilla Bernasconi

1.ii GIVIMP

Interactive session with test methods developers and session participants on in-house validation of *in vitro* methods. The goal is to exchange information on and experience with the process of in-house validation and discuss the applicability of existing guidance (or lack thereof). **(Afternoon session only)**

Facilitators: Sander van der Linden, Mounir Bouhifd, Gerard Bowe and Roman Liska

2. Miniaturised Ames *in vitro* method

A practical session in the EURL ECVAM GLP test facility, organised by the volunteering EU-NETVAL test facility VITO NV in collaboration with Xenometrix, to demonstrate differences/advantages/issues between the OECD TG 471 conventional Ames test versus the miniaturised–Ames.

Facilitators: Emilio Mendoza and Federica Madia

2nd day: 11th October 2017 continued

3. Practical aspects of test system management

EURL ECVAM will detail the test system management used for the validation of the AR-CALUX *in vitro* method as an example.

Two invited experts Glyn Stacey (National Institute for Biological Standards and Control, UK) and Jan Van der Valk (3R Centre Utrecht Life Sciences, NL) will give details about good practices for cell authentication and for use of defined serum free media alternatives.

Facilitators: Lotta Bostroem, Sandra Coecke, Susanne Belz and Donatella Carpi

4. Practical aspects of test item management

EURL-ECVAM staff will give a presentation on its procedures for the distribution of test items, coding of test items and solubility testing (both visual and with the nephelometer), using examples from validation studies.

Participants to the session will share knowledge on their in-house practices. Furthermore, interested participants are invited to a demonstration with the nephelometer in the laboratory.

Facilitators: Tom Cole, Ingrid Langezaal and Salvador Fortaner Torrent