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 7-8th May 2019, Ispra, Italy

The fifth meeting of the European Network of Laboratories for the Validation of Alternative Methods (EU-NETVAL) was held on 7-8th May 2019 (the agenda is included in Annex II).

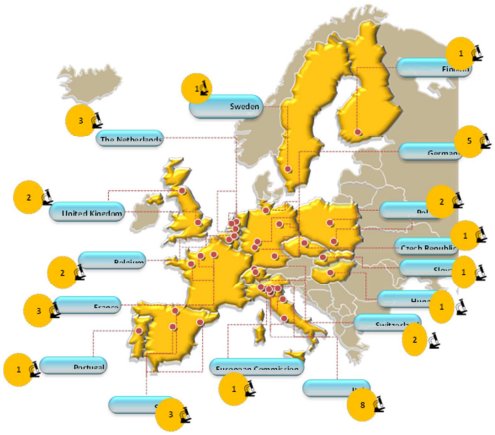
Welcome and introductory session (EURL ECVAM)

EURL ECVAM welcomed all EU-NETVAL test facilities and invited experts to the JRC.

Recent happenings in the field of complex test systems used as a modern safety assessment toolbox were presented, including among others, a new European organ-on-a-chip society and how to use these advanced *in vitro* and *in silico* models in decision making to support read across and so decrease animal use. It is envisaged that computational models will be taken up more widely in global guidelines in the near future, where mechanistic thinking will be necessary in order to pull together all these methods in a meaningful and relevant way. This win-win-win concept, with a decrease in animal testing, a better protection of people and the environment and an increase in innovation, can be achieved.

History, updates and overview of the aims of EU-NETVAL (EURL ECVAM)

At present there are currently 37 test facilities in EU-NETVAL, however DG ENV continues to stimulate missing EU-Member States to join the network. EU-NETVAL, was established in October 2014. More emphasis will be placed in the future on ensure participation of all facilities, taking into account human resources, financial capacities, and expertise. Funding of the network was also discussed, as only 5 test facilities present at the meeting indicated they received funding from their government.



In 2016, there was knowledge exchange on best practices on *in vitro* methods with training on skin sensitisation methods. There also was practical training and knowledge sharing on the mini-Ames test. Past meetings included colleagues from DG GROW (GLP issues), DG ENV (Directive 2010/63) and OECD (test guidelines).

The tasks of EU-NETVAL were presented: definition and description of *in vitro* methods; transfer of *in vitro* methods between laboratories; assessment of the reproducibility of *in vitro* methods; assessment of the predictive capacity and applicability domain of *in vitro* methods; guidance documents and training materials supporting validation (e.g. the GIVIMP which was published in September 2018); surveillance of uptake and use of validated *in vitro* methods.

Finally the agenda of the meeting and break out groups was presented; (i) the challenges ahead, (ii) how to embark on complex test items, test systems and detection systems & technologies and (iii) what we understand to be a complete *in vitro* method for regulatory use.

Updates on AR-CALUX *in vitro* method validation study (EURL ECVAM, BDS, CiToxLAB, Envigo, and RISE)

The results from the AR-CALUX validation study, experimentally executed between 2014 and 2018, were presented by EURL ECVAM. The AR-CALUX validation study was the first study completed by EU-NETVAL. Four laboratories participated in the study: CiToxLab, Envigo, RISE and BDS.

The reproducibility of the method, within and between laboratories, was found to be comparable to (or lower) than what is reported for similar assays. Also, for the first time in a test guideline, a specificity control, to check for non-specific activity (e.g. due to cytotoxicity or other interference with the luciferase production), was incorporated in the antagonist assay.

The validation study is now finalised and the validation study report (VSR) completed. The validation management group (VMG) concluded on a successful outcome. The ESAC peer-review is ongoing and the finalised opinion is planned for June 2019. The VSR, the SOPs and the ESAC Opinion will be uploaded in late summer 2019 to the TSAR website (Tracking System for Alternative methods towards Regulatory acceptance). A draft Test Guideline (TG 458) will be delivered by EURL ECVAM to the OECD in July 2019. The adoption of TG 458 by the OECD WNT is expected for April 2020 and publication by the end of 2020.

The AR-CALUX method developer, BDS, and the EU-NETVAL test facilities provided feedback on the validation study. There was overall satisfaction with the interactions of participant laboratories and BDS with EURL ECVAM. As a follow-up it was recommended to test materials (e.g. glass and plastic ware) for background activity due to the high sensitivity of the method and it was also suggested to make available the standard calculation spreadsheets in the TG. Emphasis was drawn to the need for good training, to reduce variation between operators (e.g. good pipetting practices). BDS welcomes questions regarding the procedure from the participants of the meeting.

It was underlined that knowledge transfer from one validation study to another is very important. In this case, lessons learnt from the Androgen Receptor- and Estrogen Receptor-CALUX assays will be taken into account in the Thyroid Validation study, coordinated by EURL ECVAM.

It was pointed out that method developers, when submitting a TG to the OECD in the future, will need to provide a declaration regarding protected property.

Update on OECD defined approaches: skin sensitisation *in vitro* (EURL ECVAM, OECD)

EURL ECVAM together with the OECD presented the OECD project for the development of international standards on defined approaches for skin sensitisation, as non-animal substitutes for the LLNA (OECD 4.116). The project began in 2017. In 2018, an OECD expert group was created and four expert subgroups were established to address specific issues related to the project such as applicability domain characterisation, *in vitro* variability propagation, *in vivo* variability and *in silico* models. It was noted that regulatory requirements for skin sensitisation are not harmonised

between OECD Member Countries (Casati et al., 2017) and this has to be taken into account in developing international standards.

Assessment of skin sensitisation hazard and potency categorisation requires the integration of data from various lines of evidence, as OECD adopted *in vitro* methods and are not considered one-to-one replacements. To avoid expert judgment in weight-of-evidence assessments there is the need to have a fixed combination of methods, i.e. defined approaches (DAs), translated into OECD Guidelines. DAs integrate data generated from various information sources (*in chemico, in vitro*, *in silico*). Their accuracy, specificity and sensitivity are comparable to the Local Lymph Node Assay (LLNA).

At a Joint Meeting of the IATA Case Studies Work Group and OECD QSAR ToolBox Management Group in November 2018, the use of i*n silico* data in DAs and coverage under MAD was discussed. The OECD QSAR toolbox is proposed for inclusion in one of the skin sensitisation DAs. As GLP compliance is not requested today by EFSA and ECHA as European receiving authorities for *in silico* data, criteria for adequate quality assurance had to be defined.

It is expected that there will be more defined approaches test guidelines expected in the future (eye irritation, skin corrosion).

Casati, S., Aschberger, K., Barroso, J., Casey, W., Delgado, I., S. Kim, T., Kleinstreuer, N., Kojima, H., K. Lee, J., Lowit, A., K. Park, H., J. Régimbald-Krnel, M., Strickland, J., Whelan, M., Yang, Y., & Zuang, V. (2017). Standardisation of defined approaches for skin sensitisation testing to support regulatory use and international adoption: position of the International Cooperation on Alternative Test Methods. *Archives of Toxicology*, *92*. https://doi.org/10.1007/s00204-017-2097-4

Update on the OECD detailed review paper on miniaturised Ames *in vitro* method(s) (EURL ECVAM)

EURL ECVAM provided an update on the OECD detailed review paper on the miniaturised Ames *in vitro* method(s). The requirements for genotoxicity testing across sectors (e.g. cosmetics, chemicals, biocides, Plant Protection Products, veterinary and human medicinal products) differ, and therefore the *in vitro* tests should cover 3 endpoints; point mutation, structural, and numerical chromosome aberration. A battery composed of the Ames test (OECD TG 471) and the *in vitro* micronucleus test is often recommended. Several versions of the miniaturised Ames test are also available but do not conform to OECD Test Guidelines, meaning that regulatory agencies are not bound to accept the results. In addition, they indicated that there are some concerns that these have lower sensitivity in detecting *in vivo* genotoxicity when compared to the standard Ames test. This was the reason for the initiation of an OECD project aiming at revising the available versions of the miniaturised tests, defining their accuracy and reliability and summarising these results in a detailed review paper. This analysis will serve as the basis for recommending either the development of a stand-alone test guideline or, the incorporation of the miniaturised versions of Ames test into the current OECD TG 471. The mini Ames test results are being compared to the standard Ames test.

EURL ECVAM maintains a curated reference database of genotoxicity and carcinogenicity data for Ames positive chemicals, which will be updated to include Ames negative chemicals in the near future.

It was commented that some versions of the mini-Ames (commercial kits) are very expensive, especially in a high throughput screening context.

Updates on training and dissemination: European Parliament Pilot Project and E-learning activities (including GIVIMP) (DG Environment and EURL ECVAM)

One million euros has been made available to DG Environment to promote alternatives through information sharing and education activities. This will be used to promote the uptake of existing alternatives, to help development and validation of new alternatives, to foster the exchange of information, knowledge and best practices and to provide tools for education and training to facilitate the application of the 3Rs. Six interactive training modules will be developed, based on detailed learning outcomes (theoretical knowledge and practical skills) concerning Directive 2010/63/EU *on the protection of animals used for scientific purposes*. The interactive training modules will cover: project evaluation; design of procedures (two levels); implementation of the severity assessment framework; best practice on searching for alternative methods; developing reliable and relevant *in vitro* methods and approaches (including also information on the GIVIMP). The content will be available free of charge for any individuals or course providers to be used as stand-alone training tools or as part of a curriculum.

Under the EP Pilot Project €300.000 were attributed to the JRC to develop new resources for high schools, universities, and early career scientists. The project kicked off in September 2018, and includes as deliverables learning resources and specifications for building guidance on how to include the 3Rs in a curriculum. The importance of starting education on alternative methods already at primary school level was highlighted during a scoping phase involving experts in 3Rs and education. As a sub-project European Schoolnet (www.eun.org) will bring educators together to co-design and co-create resources in their *future classroom lab* aimed at high school students. The teaching materials will be translated into the teacher's own language and the resources will be hosted on the STEM (Science, Technology, Engineering and Maths) education and training platform scientix.eu (funded by H2020). In addition, EURL ECVAM is collaborating on two of the six e-learning modules, on best practices for searching for alternatives and developing reliable and relevant *in vitro* methods for regulatory use (ref GIVIMP), to be developed by DG ENV. EU-NETVAL laboratories were invited to contribute to GIVIMP e-learning modules.

The German Federal Ministry of Education and Research runs the project 3R-SMART with training modules on alternatives and videos of various assays. It was also stated that the e-learning activities under the EP project are distinct from the technical trainings offered by the Altertox academy.

Update on EURL ECVAM, OECD and EFSA activities for Developmental Neurotoxicity (EURL ECVAM, OECD)

EURL ECVAM presented Alternative Approaches for Developmental Neurotoxicity (DNT) Evaluations. Currently, 1 out of 6 children suffers from neurodevelopmental disorders. The literature (epidemiology, *in vivo*, *in vitro*) shows a possible strong contribution of the exposure to environmental chemicals. Testing for developmental neurotoxicity is not a standard requirement and when performed, the studies rely entirely on animal testing (OECD TG 426). EFSA and US-EPA came to a consensus statement that children are at an unacceptably high risk of developing

neurodevelopmental disorders and that there is a need for new approaches to test developmental neurotoxicity using *in vitro* assays. A battery of 22 *in vitro* tests, anchored to key neurodevelopmental processes and key events identified in existing AOPs relevant to developmental neurotoxicity, has been identified. In order to make these new approaches available as soon as possible, a fit-for-purpose validation is required, where EU-NETVAL laboratories are invited to contribute to the project.

Other OECD projects with relevance to developmental neurotoxicity were described by the OECD, including a detailed review paper on retinoid signalling pathway (led by Sweden), a detailed review paper on stem cell assays for developmental toxicity (led by Japan) and activities at the OECD for the thyroid validation study.

Updates on EURL ECVAM activities for the thyroid validation study

Introduction (EURL ECVAM)

The current status of the EURL ECVAM coordinated thyroid validation study was presented.

In 2014, the OECD published a Scoping Document (No. 207), which identified *in vitro* and *ex vivo* assays for the identification of modulators of thyroid hormone signalling. The scoping document outlines how new methods can help in understanding the mechanisms behind the thyroid disrupting potential of chemicals. The scoping document divided the thyroid signalling pathways into eight blocks representing a specific mechanism.

In the validation study, 17 *in vitro* methods (at least one method per block), were selected. Human derived test systems and methods suitable for high throughput approaches were preferred. The validation study is divided in 2 parts: (1) definition of the *in vitro* methods, (2) relevance of the *in vitro* methods. Fourteen EU-NETVAL test facilities along with 13 method developers are participating in the thyroid validation study, demonstrating the capacity of the network. The importance of the contribution of the method developers in the development, troubleshooting and knowledge sharing was emphasised.

Legal agreements and Intellectual Property Rights (EURL ECVAM)

An update of the current legal agreements required for the validation study was provided. A number of legal documents (declarations, material transfer agreements and collaboration agreements) have been put in place to facilitate the interactions between EURL ECVAM, method developers, cell line suppliers and EU-NETVAL test facilities. Out of a total of 36 required, 32 are already in place and 4 are being discussed.

In April 2019, OECD adopted a document "Guiding principles on good practices for protected elements in TGs". This document states that a declaration regarding intellectual property (IP) will need to be completed and signed by method developers when submitting a new TG. To this end, protected elements related to the 17 *in vitro* methods are being investigated and documented. It was also highlighted that the new OECD standard project submission form includes new elements, such as information on the protected elements of a method, which need to be provided.

It has been quite challenging to retrieve and document the information regarding protected elements for the thyroid validation methods. The process will continue in 2019/2020.

Test system management and GMO notification (EURL ECVAM)

Nineteen test systems are included in the thyroid validation study. The relevant test system(s) will be provided by EURL ECVAM, including purchasing, shipments and quality control and if required information and documentation on test system handling and GMO notification. To date, 14 of 21 shipments have been completed. Upon receipt of the test system(s), EU-NETVAL facilities are expected to create a master cell bank and a working cell bank (if applicable), to prepare SOPs for maintenance and handling of the test system(s) and to generate historical datasets. In parallel to the experimental phase, EURL ECVAM, EU-NETVAL, the method developers, and the test system suppliers must all contribute to ensure a complete test system characterisation is made available by the end of part 1. It was underlined that notification of genetically modified test systems is not performed or requested equally in all EU Member States and that there is possibly room for better harmonisation of GMO notifications among members. In France, to avoid dispersal of GMOs into the environment, it is strongly recommended to handle them in level 2 biological safety premises.

Reference/control items management (EURL ECVAM)

The current status of the reference and control items procurement and shipment was presented. After acquisition from commercial suppliers, the reference and control items were aliquoted, labelled and shipped to the relevant EU-NETVAL facility for detailed in part 1 of the thyroid study. Inventories, material safety data sheets and certificates of analysis are provided. So far 65 chemicals (mainly reference and control items) have been identified for 10 of the methods. For part 2 of the project, chemical selection is ongoing. A list with 171 potential chemicals is being refined on the basis of specific information and in collaboration with experts. For part 2, the finally selected chemicals (test items) will be distributed to EU-NETVAL as coded chemicals.

Outline protocols (EURL ECVAM)

The current status of the outline procedures, as detailed in the original call, was discussed. The outline procedure(s), provided as a word document for easier use and modifications, is a compilation of available information written in a stepwise fashion, which can be used to create SOPs. An initial assessment of (i) the completeness of the method and (ii) the presence of acceptance criteria revealed great disparities. Acceptance criteria (when available) were assessed for each method and will be completed (where necessary) in collaboration with the method developers and EU-NETVAL laboratories.

1a – Thyrotropin-releasing hormone (TRH)-Receptor activation of pituitary thyrotropes (EURL ECVAM)

EURL ECVAM on behalf of ISZLER provided a status on this method. The method is already commercially available for drug discovery and development. For the thyroid project, the plate layout was revised from 384 to 96- well format, reference and control items were defined and data analysis and acceptance criteria were further developed.

1b – TSH receptor mediated activation (EURL ECVAM)

EURL ECVAM on behalf of the National Reference Laboratory for Experimental Immunotoxicology provided a status update. The EU-NETVAL facility has received the control cell line (CHO-K1 cells) and all available information on the genetically modified cell line containing the TSH receptor. An outline protocol has been drafted based on what is available in the reference publication. For the development of SOPs by EU-NETVAL, further details will be discussed with the method developer.

2a – TPO Inhibition assay: AUR-TPO (RISE)

The method has been developed using TPO from rat/pig. It is now intended to use cell lines producing human TPO, which have been identified (FTC-238 hrTPO and Nthy-ori 3-1 cells). The challenge now is to have sufficient dynamic range. Training has as yet not being performed.

2b – Guaiacol/Iodide oxidation/Tyrosine iodination TPO inhibition assay (VITO)

The specificity of the method is being checked using non-thyroid cell lines. Other technical optimisations are being considered: reagent, time conditions, plate layout, quality and validity criteria, templates for experimental conditions and data calculation. Quality and validity criteria are being developed. Attention was drawn to false positives due to direct interaction with TPO without crossing the membranes or due to oxidation from other molecules than TPO. The experimental work has not yet started.

2c – TPO inhibition – tyrosine iodination assay (Charles River)

Charles River gave an update of the experimental work carried out.

Cell lines have been banked, and TPO extracted and quantified as commercially available human TPO is very expensive. Differences in enzyme levels in batches could be determined with the luminol assay (method 2b). The tyrosine iodination assay seems to work well to measure iodination activity, however non-enzymatic oxidation is mainly observed. Although, positive control items were already selected and tested by Charles River, the final set of control items will be selected by the method developer. This set will also include negative controls. A prediction model is not available yet, but could potentially be similar to other enzyme inhibition assays e.g. as defined in the aromatase guideline.

A UPLC-MS/MS method has been set up to measure tyrosine and its metabolites (monoiodotyrosine (MIT) and diiodotyrosine (DIT)).

2d – Non-Radioactive Sodium/Iodide Symporter (NIS) Uptake based on Sandell-Kolthoff reaction (EURL ECVAM)

EURL ECVAM provided a status update on behalf of the EU-NETVAL TF Labfit. This *in vitro* method measures the Sodium/Iodide Symporter (NIS) activity, recognised as an important mechanism to be assessed. The outline procedure is complete. The Sandell-Kolthoff reaction, works as described in the original papers (Waltz et al. 2010) and (Hallinger et al. 2017), with comparable calibration curves produced using a series of concentrations of NaI. Preliminary testing of NIS activity was conducted on rat thyroid follicular cells (FRTL5) cells. However, significant iodide uptake into the FRTL5 cells was observed only after incubation using high concentrations of NaI. This observation was confirmed using a radioactivity-based approach. Analysis of qPCR confirmed that the level of expression of NIS in FRTL5 cells is rather low (with Ct value of about 35.79), and it was concluded that such a test system does not seem suitable to measure NIS activity. Therefore, it will be necessary to use a different test system, which expresses human NIS at a higher level.

3a – TTR/TBG 8-anilino naphthalene sulfonic acid ammonium salt (ANSA) fluorescence displacement assay (EURL ECVAM)

In the presence of T3, T4 or an unknown disruptor, ANSA (8-anilino-1-naphthalenesulfonic acid ammonium salt) is displaced from human serum protein transthyretin (TTR) or thyroxin-binding globulin (TBG), leading to a decrease in fluorescence. Among the 4 protein-ligand combinations, T4/TTR is suggested to be used as the reference. Indeed, T4 is mechanistically more important and TTR more relevant because TBG functions merely as a buffer for TTR, which transports T3 and T4 over the placenta. The next steps include the simplification of the protocol and the choice of positive and negative control items.

3b – TTR binding with fluorescent FITC-T4 (RIKILT)

The method was successfully performed as described by the method developer. Modifications were made in order to be able to use this assay also as a high throughput method. The incubation time was decreased from 2 hours to 5 minutes. The TTR/T4-FITC ratio was adjusted in order to use less label. The effect of temperature (ice versus room temperature) will be investigated.

4a – Colorimetric method for assessing deiodinases activities based on Sandell-Kolthoff reaction (Charité)

Charité provided a status update on behalf of the EU-NETVAL TF BASF. Hands-on training was performed in November 2018. The outline protocol was submitted by EURL ECVAM to BASF and Charité in April 2019. Test system (recombinant enzyme *versus* human microsomes) and reference

item were discussed. The reproducibility assessment in human microsomes will be performed in Q2-3 2019 by BASF who will do some additional development work in close collaboration with Charité. Appropriate prediction model, cut-off criteria for positive and negative substances and definition of a reliable prediction/classification model need to be considered.

4b – Chromatography/mass spectrometry (LC/MS) glucuronidation assay (Accelera)

4c – Inhibition of thyroid hormone sulfation assay (Accelera)

In vitro methods 4b and 4c do not have specific identifiable method developers, as the technique used has been widely published. The EU-NETVAL facility Accelera therefore will fulfil the role of both developer and test facility. The methods will be developed to measure only inhibition, and not induction. Given the early developmental stage, preliminary tests are yet to be performed and the test systems also need to be selected. The use of whole cells for 4b and subcellular fractions for 4c is proposed. It was felt that cells are more physiologically relevant but subcellular fraction is good enough when looking at inhibition alone. Both T3 and T4 will be tested to start with. Positive control and reference inhibitors are already selected. IC_{50} will be determined as an endpoint. It was suggested to remove propofol from the list of possible test items since it is a narcotic.

5a – T3/T4 cellular uptake assay non-radioactive (based on Sandell-Kolthoff) (Charité and Instituto de Salud Carlos III)

The outline protocol has been established and was shared with the EU-NETVAL facility in February 2019. Silychristin was found to be a potent MCT8 inhibitor. The EU-NETVAL facility, who already received the test system, will soon start the assessment of this method upon receipt of the reference and control items.

6a – Human TRα and TRβ reporter gene assay (VitroScreen)

The TR-α / TR-β reporter method will be used to assess chemicals for thyroid hormone receptor activity. Further test system characterisation will be needed (cell identification, contamination) and documented (product sheet, certificate of analysis, genetic modifications, GMO class). T3 is suggested as the reference item. Vitroscreen must assess if testing a single concentration is sufficient, or if multiple concentrations or range finder experiments must be generated. The experimental phase will start in September 2019.

6b – Thyroid Receptor (TR) β reporter gene assay (BDS and EURL ECVAM)

BDS and EURL ECVAM provided a status update on behalf of the EU-NETVAL member, VITROX ARPA ER. The test system and the reference and control items are ready to be shipped. The SOPs already exists given that is very similar to the recently validation AR-CALUX method. BDS has already performed an in-house validation of the TR-CALUX which has been accepted for publication. VITROX will be trained at the BDS facility so that the technology can be transferred to the EU-NETVAL facility. BDS is considering working with frozen cells, plating them and directly performing the analysis. The LDH leakage test will probably be substituted by another cytotoxicity/cell viability assay. It was noted that there are few positive chemicals identified for this assay.

7a – Zebrafish Eleutheroembryo Thyroid Assay (Iszler)

Training is scheduled with the method developer (Demetrio Raldua, Barcelona). Water solubility was identified as a critical point for chemical selection. Since many of the chemicals are very hazardous, there is collaboration with the prevention and protection service of the facility (identification of suitable equipment for handling substances and appropriate personal protective equipment).

8a – T-screen assay using GH3-cell line (NIOM)

It was underlined that the T-screen assay endpoint is non-specific, and therefore, there may be several false positive compounds (interference with cell proliferation of pituitary cells through a TH-independent mechanism). The first draft version of the outline procedure was received in April 2019. The next steps include preparing a master cell bank and a working cell bank, drafting/editing of a set of SOPs and assessing the within laboratory reproducibility.

8b – Human neural progenitor cells (hNPCs) assay (EURL ECVAM)

EURL ECVAM provided a status update on behalf of the EU-NETVAL member, Labfit. Method 8b is based on the use of neural foetal progenitor cells, proven suitable to measure both proliferation and differentiation towards oligodendrocytes. As the use of foetal progenitor cells may generate ethical concerns, neurospheres derived from human induced pluripotent stem cells (iPSCs) could be considered as an alternative test system, however their differentiation capacity into oligodendrocytes is rather low and further optimisation and standardisation of the differentiation protocol is needed. For these reasons, the test method is deemed not ready for regulatory application, and this method is currently on hold.

Day 2

Introduction to the interactive knowledge sharing sessions on regulatory *(mutual)* acceptance of alternative methods and the generated study data (EURL ECVAM)

During the second day, the following questions were addressed: how to deal with complex test items and complex test systems (human induced pluripotent stem cells, organ-on-a-chip, etc.)? How to get these methods to the regulatory level? How can the current test guidelines be used for new challenging test items?

In vitro evaluation of skin irritation of medical device extracts: from OECD Test Guidelines to ISO standard (Christian Pellevoisin)

Invited expert Christian Pellevoisin presented a case study using reconstructed human epidermis (rHe) for *in vitro* biocompatibility of medical devices (ISO 10993-10) for skin irritation. Medical devices are a large and complex market with lots of different systems, various materials, and various treatments which makes it difficult to regulate. According to ISO 10993-10, cytotoxicity, skin sensitisation and skin irritation assays must be performed on the finished product. At present there are only Test Guidelines for skin irritation for chemical products available. It was decided to adapt the existing and robust OECD TG 439 on reconstructed human epidermis (RHE) to an ISO standard to be used for medical devices. The Round Robin validation study (2013-2017) employing 17 laboratories and two RHE models (SkinEthic RHE from EPISKIN and Epiderm from Mattek) was a success with reproducibility and predictivity values close to 100%. The importance of having two solvents for the extraction step was underlined. Positive controls were generated by spiking medical devices materials (polymers) with irritant chemicals. The new ISO standard is expected to be available in the beginning of 2020. This success is paving the way for other endpoints (i.e. eye irritation, skin sensitisation). The initial ISO 10993-10 has been split and there is now a proposal for a new ISO standard for *in vitro* irritation testing of medical devices (ISO 10993-23). The use of 3D cell models seems to be adequate because they allow the testing of medical devices extracts in polar and non-polar solvents and react to low concentrations. Based on historical results for dermatological and cosmetic finished products where similar protocols have been used, this test could be also suitable for testing non-extractable medical devices such as liquids, gels or creams.

It was also specified that the ISO standards require for chemical characterisation of the extracts. Lastly, it was pointed out that according to Directive 2010/63/EU on the protection of animals used for scientific purposes and ISO 10993-2 for “Animal welfare requirements", *in vitro* methods must be used when available.

GLP and complex detection systems, test items and test systems: a current reality? (Thomas Lucotte)

Invited expert Thomas Lucotte, GLP inspector, ANSM (French National Agency for Medicines and Health Products Safety) appointed by DG GROW, stated that OECD *in vitro* Test Guidelines are not always applicable to complex test items. The OECD Guidance Document 19, Management, Characterisation and Use of Test Items, in which medical devices have been mentioned for the first

time in an OECD GLP document, provides guidance on the maintenance and characterisation of different types of test items that are used in the conduct of a broad range of non-clinical studies carried out in compliance with the Principles of GLP. There is at the moment no obligation to perform non-clinical safety studies of medical devices under GLP in European Union, it is however recommended. It is mandated by regulation in Japan and the USA.

Nanomaterials and UVCBs substances are not mentioned in Guidance Document (GD), nevertheless, new cosmetic reagents are mainly Chemical Substances of Unknown or Variable Composition, Complex Reaction Products and Biological Materials (UVCBs) which are addressed in the GD. Problems related to the large diversity of this group of compounds was raised, where a case-by-case approach was recommended. OECD Test Guidelines may be adapted for other test items not covered in the original TG, but it should be validated first. Furthermore, test items need to be characterised, including homogeneity, concentration and solubility data. Thomas Lucotte, highlighted that there is often a lack of analysis of possible interferences between the test item and the detection system.

Regarding complex test systems, they must be fit-for-purpose and the functionality of the test system should be assessed regularly (e.g. each new test system batch).

Detection systems are often declared as computerised systems and should be validated according to OECD Series on Principles of GLP Number 17. It has, however, been found that during GLP inspections insufficient computerised system validation was performed and insufficient management of data integrity (from generation to archiving) occurs. It was stated that the computerised system should have an audit trail function and provide for the protection of data from uncontrolled modification.

There is currently no test facility using transcriptomics for which GLP accreditation has been given in the European Union. These next generation technologies generate several terabytes of data which cannot be stored in a "normal" computer. The topic of complex detection systems, cloud systems and transparency will be discussed at OECD level. Sharing information on the development of these new complex computerised systems and their validation within the community, though challenging may be feasible at certain levels.

In the GLP OECD Working Groups issues, such as the use of cloud systems for data, are currently being discussed.

Specific interactive knowledge sharing sessions

Complex test items

The aim of this session was to exchange information and experience regarding complex test items, such as mixtures, medical devices and nanomaterials. Many of the EU-NETVAL members have experience with such items but also with (cosmetic) products, environmental samples and vegetal extracts. However, it became clear that there are many questions and difficulties to deal with and solutions are not always easy to identify.

Regarding medical devices (MD) it was emphasised that this is a group of very diverse articles, some including even software. The definition of medical devices is not harmonised between the various jurisdictions (EU, US) which has an impact on the testing requirements. Typically, an extract from a MD is produced and used for testing purposes. There was agreement that it is key to obtain an extract that appropriately represents the product and is stable throughout testing and storage. Extracts from MDs should ideally be well characterised but a minimum of properties, such as pH, osmolality, appearance, presence of undissolved matter, should be known. When extracting medical devices, mostly following ISO guidelines, one should be aware that due to the dissimilarity of medical devices due to process variability (even from the same batch) data might show variability. In the case of MDs that have similar matrices as cosmetic products, both ISO and OECD Guidelines are used for testing. Some laboratories use the product itself and the extract for testing, but it has to be kept in mind that the *in vitro* OECD TG methods used are not validated for product testing and thus it is the responsibility of the test facility to ensure these methods are validated in-house.

Currently there are no approved test guideline methods for the testing of nanomaterials (NM). A particular difficulty of testing NM is the dosing and it is important to also assure that the material is in full contact with the test system. Depending on the properties of the NM, such as particle size and shape, the sedimentation/floating behaviour may vary. Determination of the effective concentration is also challenging. In addition, the results of testing are influenced by the portion of NM incorporated into the cells of the test system or bound to its membrane. Measurement of remaining NM in the exposure medium may be used to estimate the effective concentration.

For environmental samples and vegetal extracts no standard methods are available and it needs to be taken into account that for environmental samples the sampling method may impact the test item (sample) composition. The composition of such samples/extracts is rarely known and solubility as well as stability during storage may be an issue. Often laboratories test both, the obtained extract/sample and a dilution of it. This gives more comprehensive information but interpretation can be challenging if the results are different for concentrated *versus* diluted item.

As OECD test guidelines are not validated for products (i.e. mixtures) but only for single substances, validation needs to be done by the test facility for each type of product. While providers of test systems may declare that a OECD TG method is also applicable for (a certain type of) products or mixtures, validation data are normally not communicated. Thus it is the responsibility of the testing laboratory to validate the method accordingly. Still, it needs to be kept in mind that a test system may not be compatible with a 'product' in particular when long incubation times are used. It was proposed that a solution could be to test a surrogate of the product, e.g. an extract, which however should be as close as possible related to (and representative of) that product. However, besides validation of such a procedure, a scientific justification should also be provided. In addition, suitability of the test system could be assessed by a 'spiking method': A known positive

item/substance is added to the product/surrogate, tested and results of spiked and not spiked items are compared. This can be repeated for different concentrations and by using other known positives of different potency.

Complex test systems

For the purpose of the session, a complex test system was defined as: "An *in vitro* cell-based 2D or 3D model usually composed of more than one cell type for which interaction is required to be relevant to human biology, based on the fit-for-purpose principle". The wording "complex" is open to interpretation, e.g., human induced pluripotent stem cells or organ-on-a-chip would fall under the definition.

Using a more complex test system does not automatically mean it is more relevant for humans, it depends on the purpose. Cells in 3D culture have different responses, differentiation and communication. For bioavailability tests the 3D models are relevant, however, when a 2D model is predictive and can provide a specific answer, there is no need to select a complex test system. The endpoint should be the driver for the selection of the complex test system to mimic the human physiology. The participants mentioned that, the selection of a complex test system, rather than a 'simple' 2D model, can be preferred:

- to increase the number of applications
- to increase the applicability to more regulatory fields
- to increase data translatability
- to better mimic the exposure conditions and functions/physiology/interactions in tissues
- to reproduce the microenvironment

Increasing confidence in complex test systems can be achieved by demonstrating that:

- The function of interest and response/sensitivity is maintained over time, with historical data from positive and negative control chemicals and cell specific quality control. Also test system suppliers should generate and report historical data.
- The system has biological relevance for humans. Functional capacity should be shown.
- Production is standardised and there is low batch to batch variability (using appropriate biomarkers/characterisation)
- Good quality instructions from the supplier. Procedures/SOPs exist including details about batch to batch variability, possible limitations, use of specific material or equipment and critical steps (published papers are not enough)
- The model is transferable to laboratories
- General considerations in GIVIMP regarding contamination, authentication, etc. are addressed

Different quality controls are needed during the production phase (e.g. viability, permeability, histology) and experimental phases.

It would be beneficial to harmonise and standardise the characterisation (morphology and functions) and develop a defined list of biomarkers and their levels of expression. Harmonisation of the characteristics and functional endpoints of the test system is needed (not the exact protocols), and will be possible once the system is validated for a specific purpose.

Validation of a (complex) test system is currently done as part of the establishment of a test guideline, as it is used for a specific application/purpose. However, it would be beneficial when

complex *in vitro* test systems could be qualified and characterised (using its biomarkers) without being part of an *in vitro* TG method. The same qualified complex test system could then be integrated into different *in vitro* methods adding thresholds for specific functions/endpoint, e.g. the epivaginal model was not validated for estrogen disruption, but is a promising model for that endpoint. Likewise, it would be beneficial when different test systems with the same function but different thresholds can be used in the same test method. Validation of the test system is desirable, but may not be achievable; however qualification (usually smaller in scope than validation) of the test system is advisable.

A complex *in vitro* test system is ready for regulatory purpose/consideration when it is fully characterised (demonstrated by data, detailed in SOPs, etc.), the endpoint measured is relevant and the results are reproducible. The more a test system is characterised, the less testing is needed during ring trials. A standard (or benchmark) would be needed for the characterisation of *in vitro* models.

Complex detection systems

The aim of this session was to exchange information and experience among EU-NETVAL members on complex detection systems, such as high content imaging, omics and robotic platforms. Complex detection systems and technologies produce vast amount of data and therefore require sophisticated data processing strategies. The discussion focused on the experience of the laboratories present in the sessions.

Some of the participants have extensive experience in high content imaging, especially in relation to the Ames and Comet assays. Others have experience in liquid chromatography, liquid chromatography-mass spectrometry and gas chromatography-mass spectrometry, which they run under Good Laboratory Practice (GLP) conditions. Some EU-NETVAL facilities have experience in next generation sequencing, metabolomics, transcriptomics, and robotics. Therefore, the principles of the validation of data recording, processing, integrity check and data storage are already in place. These principles are common to different detection systems and methodologies but there is a need to transfer them to more complex detection systems. The need to validate computerised systems and to have safe and traceable data which cannot be manipulated were emphasised. Data integrity should be fully considered when using external servers.

One of the current challenges is related to data interpretation, particularly in relation to machine learning, predefinition of criteria for data interpretation (e.g. the results are not "on/off", but "upregulation/down-regulation" of genes), and in relation to the use of commercial software which do not cover the whole data analysis pipeline. Moreover, the applicability domain is not always clearly defined and the regulators might not have the full understanding of the technologies and the data generated. There is a need for more knowledge transfer from scientists to "educate" regulators in order to enhance confidence in the data presented. Collaboration with the DNA field was suggested since methods have already been accepted in that field.

One of the NETVAL laboratories shared a positive experience where metabolomics data were produced under GLP and submitted to ECHA to support waiving of animal data through read-across. It was suggested to work closely with regulators by taking a step-wise approach, in which regulators are initially exposed to omics data via workshops and presentations, then the data submission process is standardised (including biostatistical analysis), and finally, data are submitted as supplementary information in order for regulators to acquire the appropriate confidence.

Designing a method for regulatory use

The completeness of a method was discussed. It was underlined that a method is never "complete", as further experience could always lead to improvements. A method can also be considered "complete" when it is more predictive of human effects than the animal method.

The submission of a TG to the OECD should be encouraged as soon as it is "good enough", i.e. fit-for-purpose. The strength and limitations of an assay that is fit-for-purpose should always be described and the cost should not be overlooked.

A series of specific issues were discussed. Cell characterisation is an important step and it can be necessary to perform it multiple times, especially to ensure that the system is the same as initially described (after serum addition for example). Additionally, it is important to confirm that the test items are freely available to be able to act (e.g. unbound fraction) and their interaction with the detection system should be addressed. Also, the equipment needed for performing the assay and the requirements (working range, specific conditions and acceptance criteria) should be included in the method. It was agreed that the plate layout is especially important when linked to the analysis of the data analysis. The use of outer wells can be allowed depending on standard deviation values. Investigation of test item cytotoxicity is of great importance, particularly for loss-of-function assays. Threshold value and measurement methods should be described. There is a wish for a universal, reliable cytotoxicity method based on automated imaging systems.

The definition of acceptance criteria and the robustness of the assay were discussed. Critical steps in the method need to be identified and acceptance criteria need to be defined. Acceptance criteria should be defined during method development. Materials used for carrying out an experiment must be controlled before running the method and the control of the nominal dose of the test item should be checked through confirmatory measurements. These procedures need to be described in the standard operating procedure. The robustness of a method can be evaluated by identifying and assessing the acceptable variations of aspects of a method which do not affect the assay, possibly using historical data. The acceptable variability should be documented. Quality control criteria need to be defined and historical data should be produced as early as possible in the method development process.

Finally, regarding data analysis, the need for control plates and validated spreadsheets was highlighted. It was also noted that there were issues between different versions of the same software and the use of various EU languages.

While some preferred modular, stepwise SOPs which can be re-used in other methods, others prefer having a single SOP for each method. Changes in SOPs should be assessed for their impact on the method.

Annex II - Agenda

Meeting of EU-NETVAL member test facilities

JRC Ispra, 7 – 8 May 2019

1st day: 7 May 2019
Building 58c, Auditorium

09:00-09:30	**Welcome and introductory session** Overview of main activities Directorate F, F.3 EURL ECVAM - *Maurice Whelan, F3 EURL ECVAM* History, updates on EU-NETVAL and overview of the aims - *Sandra Coecke, F3 EURL ECVAM*
09:30-10:00	**Updates on the AR-CALUX *in vitro* method validation study** Results from the validation study for the Transcriptional activation assay for detection of anti-androgenic activity of chemicals (AR-CALUX) – *Anne Milcamps and Roman Liska, F3 EURL ECVAM.* ***Feedback from the method developer - Harrie Bezzelink, BDS and the EU-NETVAL test facilities – CiToxLAB; Envigo; RISE***
10:00-10:30	**Update on OECD defined approaches: skin sensitisation *in vitro*** *Silvia Casati, F3 EURL ECVAM and Patience Browne, OECD* ***Q&A session by EU-NETVAL members***
10:30-11:00	Coffee break (Building 58c, Auditorium)
11:00-11:30	**Update on the OECD detailed review paper on miniaturised Ames *in vitro* method(s)–** *Raffaella Corvi and Federica Madia , F3 EURL ECVAM* ***Q&A session by EU-NETVAL members***
11:30-12:00	**Updates on training and dissemination: European Parliament Project and E-learning activities (including GIVIMP)** - *Susanna Louhimies, DG Environment and Marcelle Holloway, F3 EURL ECVAM* ***Q&A session by EU-NETVAL members***
12:00-12:30	**Update on EURL ECVAM, OECD and EFSA activities for Developmental Neurotoxicity** *Anna Price, F3 EURL ECVAM, and Patience Browne, OECD* ***Q&A session by EU-NETVAL members***
12:30-13:30	Buffet lunch (Building 58c, Auditorium)
13:30-14:30	**Updates on EURL ECVAM activities for the thyroid validation study –** **Introduction**: *Sandra Coecke, F3 EURL ECVAM* **Legal agreements and IP** - *Anne Milcamps, F3 EURL ECVAM* **Test system management and GMO** – *Ingrid Langezaal and Camilla Bernasconi, F3 EURL ECVAM* **Test item management** – *Tom Cole, F3 EURL ECVAM* **Outline protocols**– *Gerard Bowe, F3 EURL ECVAM*
14:30-18:20	**Updates on EU-NETVAL activities for the thyroid validation study** ***Flash presentations by EU-NETVAL members & method developers (or EURL ECVAM) on interactions, training and transfer experience*** • *Camilla Bernasconi (1a, 1b) and Markéta Dvořáková - NRLEI (1b);* • *Emma Pedersen - RISE (2a); Hilda Witters - VITO (2b); Jeroen Rijk - Charles River NL, (2c); Francesca Pistollato (2d);* • *Thomas Cole (3a); Taine Bovee - RIKILT (3b);* • *Kostja Renko - Charité and Barbara Birk - BASF (4a); Flavio Cinato - Accelera (4b, 4c);* • *Kostja Renko - Charité and Francesca Pistollato, Antonio della Vieja – ISC (5a);* • *Laura Ceriotti and Marisa Meloni - Vitroscreen (6a); Harrie Besselink - BDS and Anne Milcamps (6b);* • *Maria Sampieri and Silvia Dotti - Iszler (7a);* • *Joanna Roszak - Nofer (8a), Anna Price and Rita and Anna De Oliveira - Labfit (8b)* Interrupted by a Coffee break at 16:00 (Building 58c, Auditorium)
18:20	Departure
18:45	Dinner

2nd day: 8 May 2019
Building 58c, Auditorium

09:00-09:10	**Introduction to the interactive knowledge sharing sessions on regulatory *(mutual)* acceptance of alternative methods and the generated study data**- *Sandra Coecke, F3 EURL ECVAM*
09:10-09:35	***In vitro* evaluation of skin irritation of medical device extracts: from OECD Test Guidelines to ISO standard** - *Christian Pellevoisin, Episkin Academy* ***Q&A session by EU-NETVAL members***
09:35-10:00	**GLP and complex detection systems, test items and test systems: a current reality?** *Thomas Lucotte – ANSM* ***Q&A session by EU-NETVAL members***
10:00-10:15	**1. EU-NETVAL Survey: Complex test items (e.g. mixtures, biomedical devices, nanomaterials)** *Aude Kienzler , Taina Palosaari and Susanne Belz, F3 EURL ECVAM;* *Claudius Griesinger, F2*
10:15 -10:30	**2. EU-NETVAL Survey: Complex test systems (e.g. 3D, induced pluripotent test systems) –** *Ingrid Langezaal, Anna Price, Pilar Prieto, Joao Barroso and Francesca Pistollato, F3 EURL ECVAM*
10:30-10:45	**3. EU-NETVAL Survey: detection systems (e.g. omics, image-based methods, medium/high-throughput, data & computerised systems)** *Donatella Carpi, Raffaella Corvi, Laura Gribaldo, Emilio Mendoza and Jukka Sund, F3 EURL ECVAM*
10:45-11:00	**4. Designing a method for regulatory use** *Gerard Bowe, Roman Liska, Sharon Munn , Emilie Da Silva, Sandra Coecke, Elise Grignard and Valerie Zuang, F3 EURL ECVAM*
11:00-11:30	Coffee break (Building 58c, Auditorium)
11:30-12:45	**Specific interactive knowledge sharing sessions on topics 1, 2, 3 and 4 (in building 101 rooms 1302, 2002, 2302 and F3 EURL ECVAM laboratories)** ***Q&A session by all break-outgroup participants***
12:45-13:45	Buffet lunch (Building 58c, Auditorium)
13:45-15:00	**Specific interactive knowledge sharing sessions on topics 1, 2, 3 and 4 (in building 101 rooms 1302, 2002, 2302 and F3 EURL ECVAM laboratories)** ***Q&A session by all break-out group participants***
15:00-15:20	Coffee break (Building 58c, Auditorium)
15:20-16:45	Briefing on interactive knowledge sharing sessions and **additional items raised by EU-NETVAL members**
17:00	Departure

Annex II – Agenda Continued: Breakout groups

Meeting of EU-NETVAL member test facilities

JRC Ispra, 7 – 8 May 2019

2nd day: 8 May 2019, knowledge sharing sessions to identify issues and possible improvements that will advance (regulatory) acceptance and use of complex elements in *in vitro* methods.

Aim:

1. Complex Test Items

This specific interactive knowledge sharing session aims at exchanging experience on complex test items (CTIs), such as complex substances/mixtures, nanomaterials and medical devices. This will include discussing the difficulties that might arise (such as the applicability domain of test methods, solubility issues, etc.), when dealing with CTIs, and how to overcome them. The discussion will start with, but should not be restricted to, the outcome of the survey on this topic.
Participants are invited to highlight existing issues with (regulatory) acceptance of data from complex test items and discuss if action is needed to enhance regulatory acceptance of specific CTI's and what can be done in general for regulatory acceptance.

2. Complex Test Systems

Participants are invited to highlight existing issues with (regulatory) acceptance of complex test systems (CTS, e.g. 3D tissue models, human induced pluripotent stem cells, etc.) and discuss if action is needed to enhance regulatory acceptance of specific CTS's and what can be done in general for their regulatory acceptance. EU-NETVAL test facilities and invited experts will identify aspects that need further clarification to use CTS under quality systems such as ISO and GLP.

For the discussion a complex test system is defined as:
'An *in vitro* cell-based 2D or 3D model usually composed of more than one cell type for which interaction is required to be relevant to human biology, based on the fit for purpose principle'.

The following questions will guide the discussion:
- Why and when do we need complex in vitro models? Is a model more complex always better?
- How can we increase confidence in complex test systems? Identify QC measures that increase trust in the test system.
- To what extent do we need to harmonise & standardise between laboratories using the same CTS or between CTS?
- Is validation of CTS achievable and desirable? What type of validation?
- When is a model ready for regulatory purpose?

3. Complex Detection Systems and Technologies

Complex detection systems and technologies (CDS&T's i.e. proteomics, metabolomics, high content imaging, (high)-throughput analytical methods etc.) produce vast amount of data and therefore require sophisticated data processing strategies. Although these technologies have been exploited routinely already for a long time in basic research and various industrial sectors, their use in regulatory context is still minimal. The aim of this discussion group is to facilitate knowledge exchange on the use of complex detection systems and technologies, and to identify aspects that need further clarification to use such CDS&T's under quality systems such as ISO and GLP.

4. Designing a method for regulatory use

Participants are invited to discuss the design of an in vitro method for regulatory use, which will also include discussion on setting acceptance criteria and in-house validation approaches.

The following questions will guide the discussion:
What are the elements of a method specific to regulatory use?
What are have we learned from the thyroid validation case study when we look at the definition of completeness of methods?
What elements belong in the method design and what elements belong in the experimental design?
What elements should be considered when designing a robust method?
Other