Questions and Answers from REACH Webinar 2: Skin Irritation and Corrosion

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1. The human skin model test OECD test guideline 431 described in Regulation on test methods (EG) 440/2008, B.40 BIS allows the identification of corrosive chemical mixtures. It further allows the identification of non-corrosive mixtures when supported by a weight of evidence determination using other existing information (e.g. pH, human and/or animal data). If pH of a mixture is extreme (pH is less than 2 or more than 11.5) and human data do not exist, does this mean: back to animal experiment if a mixture should not be labelled as corrosive?

   GEC: No. If the results of the OECD 431 assay (skin corrosion) are non-corrosive, the test substance should be tested next in the Skin Irritation Test (OECD 439), as per New Guidance Document on an Integrated Approach on Testing and Assessment (IATA) for Skin Corrosion and Irritation No. 203 that can be found at this link: http://www.oecd.org/officialdocuments/publicdisplaydocumentpdf/?cote=env/jm/mono(2014)19&doclanguage=en.

2. Are there any regulatory limitations on using OECD test guideline 435 (Corrositex) specifically? Can it be used to exclude from being classified as corrosive? Or only used to confirm it is corrosive?

   GEC: The Corrositex assay is used to assign UN Packing Groups to corrosives or verifies if a test substance is non-corrosive. Test substances predicted non-corrosive should be tested next in the Skin Irritation Test (OECD 439), as per the New Guidance Document on an Integrated Approach on Testing and Assessment (IATA) for Skin Corrosion and Irritation No. 203 that can be found at this link: http://www.oecd.org/officialdocuments/publicdisplaydocumentpdf/?cote=env/jm/mono(2014)19&doclanguage=en.

3. In IIVS's experience, how many substances do not qualify for the testing?

GEC/HAR/IIVS:

It might not be appropriate for IIVS or any other CRO to provide this information since 1) each CRO's experiences with their Client's chemicals and mixtures would not likely be representative of the universe of chemistries and mixtures, and thus would provide a skewed answer, and 2) these analyses would be derived from testing results which are regarded as Client's Confidential Information.

Since all assay systems have some limitations and exceptions to their applicability domains, the OECD Test Guidelines typically provide some guidance to address potential issues with specific chemistries. Regarding the qualification of test chemicals for use in the Corrositex assay, OECD Test Guideline 435 presents in ¶5: "*A limitation of the validated reference test method that is the basis for this Test Guideline is that, based on the results of the initial compatibility test (see paragraph 13), many non-corrosive chemicals and chemical mixtures and some corrosive chemicals and chemical mixtures may not qualify for testing. Aqueous substances with a pH in the range of 4.5 to 8.5 often do not qualify for testing; however, 85% of chemicals tested in this pH range were non-corrosive in animal tests.*" In a personal communication with Rich Ulmer (InVitro International, manufacturer of the Corrositex kit), they estimate that of the materials they have tested historically that had a pH within the range of 4.5 to 8.5, less than 5% failed to qualify for testing. Accordingly, users of this technology should indeed conduct the Qualification screen with their specific chemicals and mixtures to determine whether they qualify for testing in the Corrositex system.

Similarly, for skin irritation and corrosion assays conducted on reconstructed human epidermis models using the MTT viability endpoint, some chemicals (ie strong colorants and direct reducers of MTT) may interfere with the MTT endpoint. For example, in the *in vitro* Skin Irritation Test Method OECD Test Guideline 439, ¶26 presents: "*Optical properties of the test chemical or its chemical action on MTT (e.g. chemicals may prevent or reverse the colour generation as well as cause it) may interfere with the assay leading to a false estimate of viability. This may occur when a specific test chemical is not completely removed from the tissue by rinsing or when it penetrates the epidermis. If a test chemical acts directly on the MTT (e.g. MTT-reducer), is naturally coloured, or becomes coloured during tissue treatment, additional controls should be used to detect and correct for test chemical interference with the viability measurement technique.*" Although some chemicals may have the potential to not be qualified for testing because of their potential to interfere with the MTT endpoint, users of the technology should indeed conduct the assay with adaptive controls to correct for any interference, since in relatively few cases historically the magnitude of the MTT interference is so great that the test results are not qualified for further interpretation and evaluation.

4. For Corrositex, are coloured substances are not suitable?

   GEC: Colored materials can be tested using Corrositex as long as they induce a color change in to the Color Detection System (even if induced by the actual color of the test material).

5. What is the response of the RHE irritation (test guideline 439) for corrosive substances?

   GEC: In general, corrosive materials tested in the RHE irritation assay are predicted irritants. The viability values (levels) obtained in the irritation assay do not provide information regarding their corrosive classification though, and hence all materials giving positive results in the RHE Skin Irritation Test should be tested for corrosion.

6. Using RHE models, have you ever come across instances of negative results in the *in vitro* skin irritation test but positive in the *in vitro* skin corrosion test?

   GEC: No.

7. Can you provide additional details on turnaround times and costs?

   GEC: Turnaround times: in general, 2-4 weeks (3 on average) are needed to reserve the reconstructed tissues from the manufacturers. The 3D-based assays are relatively short (2-4 days maximum). The Corrositex assay is also relatively short and the kits are available within 2-3 days from the time the order is placed.

   CR: Prices are very much variable, depending on the CRO, geographical area, GLP requirements, precise type of protocol, scope and so on. More and more labs are now equipped for running the tests, and hopefully the price will be reduced in the near future. Contact your CROs for specific prices, and be sure to carefully select the most appropriate test(s) in order to minimise the need for additional testing (and extra costs).

8. Why is the *in vitro* testing more expensive than *in vivo*, bearing in mind that some of these may not even be acceptable?

   CR: *In vitro* testing requires completely different lab equipment and expertise than *in vivo* and therefore they cannot be compared directly. The assays that were presented during the webinar are all accepted. The main hurdle is the selection of the right tests, *i.e.*, that there is the correct assessment of the applicability domain and that a proper testing strategy is considered, in order to avoid confirmation with a second experiment (and extra costs).

   In addition to costs, companies are not completely deaf to ethical issues and therefore they can be convinced to use *in vitro* if the cost is the same or slightly higher than *in vivo* and you can demonstrate that the information is of higher quality. Please, consider that the text of REACH says that "testing on vertebrate animals for the purposes of this Regulation shall be undertaken only as a last resort."

9. Are *in vitro* skin tests permitted to derive specific concentration limits to be used in the additivity (= calculation) method?

   CR: Additivity method is established by CLP based on the category of the classification. For skin irritation, that is not a problem—as the *in vitro* methods do characterize enough the property.

   It is an important issue for other endpoints like skin sensitisation, and would be an interesting question to ask during the upcoming PETA International Science Consortium, Ltd., and Chemical Watch skin sensitization webinar on January 28th. For more information or to register for that webinar see here: http://www.piscltd.org.uk/reaching-alternatives-animal-testing/

10. How are *in vitro* tests vs. *in vivo* tests supported financially in general by governments in EU?

    CR: As far as I know there is no government financial support that is specific for *in vitro* vs *in vivo*, but there is minor funding for research in the area of new method development. However, this is very much variable within each country and even within regions. For sure, there is no harmonized policy from the European Commission to support and propel the application of *in vitro* instead of *in vivo* methods.

    Several NGOs dedicate financial support specifically to the development of tests that support replacement, reduction and refinements, including the PETA International Science Consortium, Ltd., the Alternatives Research and Development Foundation (ARDF), and the National Centre for the Replacement, Refinement, and Reduction of Animals in Research (NC3Rs).

11. Can the *in vitro* methods be used for assessing mixtures?

    GEC: Initially, the *in vitro* methods were designed for ingredients. However, over the years, with more data available from industry on mixtures (which are of most interest to manufacturers), the *in vitro* assays are now considered useful for mixtures, and this fact is included in the published OECD test guidelines.

12. Can these methods be applied to medical devices?

    GEC: There are two standard assays that can be performed for medical devices and that were not presented during this webinar (MEM Elution or Agar Overlay that follow USP and ISO guidelines). However, it is conceivable that extracts from medical devices can be assessed in any of the *in vitro* assays presented during the webinar possibly in a tiered approach. For more info on this topic please refer to the manuscript by Casas *et al.*, 2013 (PubMed ID 23999410), "In vitro human skin irritation test for evaluation of medical device extracts", *Toxicol In Vitro* 27(8), 2175-2183.

13. I thought H318 causes serious eye damage. Can you explain why it is an irritant?

CR: You are right: H318 causes serious eye damage while H319 causes serious eye irritation. H314 causes severe skin burns and eye damage and it is used for irreversible damage to both skin and eye and therefore the H314 labeling makes H318 redundant. On the other hand, it frequently happens that H318 (causes serious eye damage) classifies a substance in combination with H315 (causes skin irritation), *i.e.*, it often happens that the damage to the eyes is more severe than the damage to the skin.

14. Did I catch that only the EpiSkin will separate Class 1a, 1B and 1C in H314?

GEC: The OECD test guideline 431 specifies that EpiSkin™, EpiDerm™, SkinEthic™ and epiCS® test methods are able to sub-categorize (i.e., 1A versus 1B-and-1C versus NC) but differences are observed between EpiSkin™ and the three other test methods (EpiDerm™, SkinEthic™ and epiCS®) in view of their capacity to provide information on sub-categorisation. Results from EpiSkin™ can be used as such; whereas results from EpiDerm™, SkinEthic™ and epiCS® generate high over-classification rates for a combination of categories 1B and 1C (see Annex 4 of the guideline). Therefore, for EpiDerm™, SkinEthic™ and epiCS®, chemicals that are classified as 1B-and-1C can be considered as 1B-and-1C, while chemicals for which cell viability at 3 minutes is below 50% should just be considered as Category 1, since the Category 1A predictions of these three test methods contain a high rate of over-predictions of chemicals of Categories 1B and 1C.

15. For GHS classification purposes, must the test be done in the EU vs. the USA?

GEC: The assays are validated and can be performed by the laboratories proficient in conducting the assays, regardless of their geographic location.

CR: In OECD countries, there is the principle of mutual acceptance and therefore the test can be performed anywhere, provided that it is fully GLP complaint.

16. Please tell us about the false positive rate of *in vitro* tests.

GEC: The OECD guidelines present the rate of false positive and negative results as obtained for each of the methods. Please download the most updated version of the assay of interest and there you will find the % of false results (positive and negative; see here: http://www.oecd-ilibrary.org/environment/oecd-guidelines-for-the-testing-of-chemicals-section-4-health-effects_20745788). Furthermore, refer to the validation test results for concordance of *in vitro/in vivo* results.

17. What can NGO do to support the *in vitro* tests?

    GEC: PETA's Science Consortium has put forth the resources to provide an education and outreach forum as exemplified by this webinar. These activities drive eventual acceptance and use of the *in vitro* tests by industry and regulatory agencies alike.

    CR: Training and dissemination, as first steps followed by lobbying to ask for financial support from the Governments to companies that prefer *in vitro* instead of *in vivo.*

18. Several formally validated and accepted RHE models are available for the prediction of a substance's skin irritation potential. The general protocols are similar. A set of performance standards is available. From a regulatory perspective, is it acceptable to use a 'me too' RHE/MTT assay which has been internally validated and has been demonstrated to be valid based on published performance chemicals or would it be strongly advised that such an assay be evaluated formally?

    GEC: For regulatory purposes (submissions to regulatory agencies), it is advisable to use one of the assays already validated. If the class of products is not correctly predicted (see question #38), one can design an alternate protocol that best predicts the respective class and use the data for internal purposes and as weight-of-evidence (bridging) purposes even for submissions. However, it may not be sufficient from a regulatory body's point of view for a method to be "internally validated" (i.e., validated in a single lab).

    CR: Annex XI of REACH explains how non standard methods can be used for registration purposes and therefore any assays can be used even if not yet published in the Regulation 440/2008 if the scientific validity can be demonstrated, even with an internal validation process.

19. To clarify, none of these *in vitro* methods are accepted for registration from EPA, correct?

    GEC: Results from the corrosion assays discussed in the webinar are fully acceptable to EPA.

20. In your example in slide 43, the starting point was skin irritation. Could you please explain the top down approach used in this case? This is requested by REACH.

    CR: It is a combination of top down approach for eyes and bottom up approach for skin, with the support of the weight-of-evidence studies from other endpoints (physical chemical properties and QSAR). This is fully accepted by REACH and allows a reduction of the number of experimental tests that must be performed for the characterization of the substance. It must be fully justified in the dossier.

21. Given the QSAR for anisole and the BCOP data, how can you rule out the positive RHE was not a false positive for thioanisole?

    CR: Remember that RHE is a fully validated method and the result is accepted with no need for further confirmation. Risk for false positive or negative result is always present for any method, both *in vivo* or *in vitro*. In the specific example, there is no QSAR for anisole, but an *in vivo* test which returns a mild and reversible effect for irritation and a more persistent dryness. Regarding the Toolbox prediction for thioanisole, even though there is no specific alert, it falls out of the applicability domain for a final decision on skin irritation.

22. May I ask Costanza if this strategy may apply to UVCB substances such as by-products of metal industry? (e.g. slags)

    CR: That's a good question, but I have no precise answer. It is a case-by-case study. If you need that, you can contact me directly and we can develop together the most suitable strategy.

32. Is it possible to apply these strategy in waste-streams based on ingredients?

    GEC: Theoretically one can test waster-streams for corrosion/irritation hazards.

33. What can you do to teach student in natural sciences the *in vitro* methods in order to enhance the need of ending animal testing?

    GEC: The Institute for In Vitro Sciences (IIVS) has its mission to teach the *in vitro* methods currently available. IIVS holds a Practical Methods Workshop yearly (next one in early January 2015) and we have also been involved in teaching toxicology classes focused on *in vitro* methods to students from a local University. We would be happy to continue such activities for natural sciences students, to provide hands on training and lectures and to promote the *in vitro* methods.

    CR: I usually insist in the weakness of *in vivo* (animal) methods— many of them are not validated, they have a very high rate of false results, and a more advanced knowledge can be gained through *in vitro* strategies. Unfortunately, many people still believe that out of *in vivo* there is nothing.

34. Which *in vitro* method is better to classify mixtures with extreme pH (strongly acidic or strongly alkaline)? Are there tests that are not suitable at all for this category?

GEC: Extreme pH formulations can be over-predicted by RHE skin corrosion *in vitro* tests as detailed in the poster entitled "An Evaluation of the Reconstructed Human Epidermis (RhE) Method for Predicting Skin Corrosivity of Chemical Products with Extreme Acid pH" and authored by scientists at (then) Diversey, Inc. (now SealedAir) and IIVS. The poster can be found at this link: http://www.iivs.org/workspace/assets/publications/iivs_poster_an-evaluation-of-the-reconstructed-human-epidermis-rhe-method-for-predicting-skin-corrosivity.pdf.

35. Are there any specific chemistries where EpiOcular and EpiDerm would provide false positive or false negative results?

GEC: Yes. Please refer to the comments on applicability domains and limitations in the relevant test guidelines.

36. Were the *in vitro* tests validated against human data or the not-validated *in vivo* data?

GEC: The *in vitro* tests were validated against *in vivo* data that were available. Human data are available for some chemicals; however, the validation was performed against animal data which may not provide the best test system to extrapolate to human from.

37. There appears to be confusion as to which *in vitro* methods are validated for regulatory purposes. Could you please confirm which methods are validated?

GEC: All methods presented in the webinar are validated for regulatory purposes. They all have OECD test guidelines associated with them. The NTP website provides a current listing of the status of validation and acceptance of *in vitro* test methods and can be found at this link: http://ntp.niehs.nih.gov/pubhealth/evalatm/test-method-evaluations/project-milestones/index.html.

CR: First of all, if there is an official OECD guideline the assay is accepted, at least for REACH registration dossiers (each Country and each Act may have own restrictions and requirements). However, there is a lag between the end of the validation process and the publication of the OECD guideline, for example as it is the case for 3T3 Neutral Red Uptake Cytotoxicity Assay for the identification of substances not requiring classification for Acute Oral Toxicity that has already received a positive opinion by ECVAM (see https://eurl-ecvam.jrc.ec.europa.eu/eurl-ecvam-recommendations). One suggestion is to subscribe to specific newsletter like Altweb (http://altweb.jhsph.edu/newsletter/)

38. We have experience with amine chemistry of false negatives with *in vitro* skin corrosion and irritation tests. Recently published.

    GEC: the manuscript this comment refers to was recently published under open access in the Toxicology In Vitro Journal and can be freely downloaded from this link: http://www.sciencedirect.com/science/article/pii/S0887233314001994.

39. Which assay(s) would be useful in understanding a delayed response that may be seen in animals?

    GEC: The three-dimensional models currently available are limited in mimicking a delayed response that may be observed in animals. To some extent, OECD 439 protocol (Skin Irritation Test) covers delayed effect of test materials. Extrapolation to the 28 days evaluation in the animal model is rather difficult to perform. Should some materials be anticipated to have delayed effects, modification(s) of the current validated *in vitro* assays may be considered, and data used as weight of evidence for regulatory submission purposes. Consult a CRO regarding any modification of a currently validated assay that is appropriate for the goals of your project.

40. What about reversibility of effects? In recent experiments, there were some discrepancies among *in vitro* methods and *in vivo* as well.

    GEC: Reversibility effects are difficult to mimic. The current assays were initially designed for hazard identification. As industry more often raises the questions of reversibility, the topic should be further investigated. Industry, CROs, regulatory agencies should come together to discuss about how to best assess this endpoint.

41. Are there any efforts ongoing to identify delayed effects and reversibility for eye irritation endpoint?

    GEC: The PETA International Science Consortium, Ltd., and Chemical Watch host an upcoming webinar on eye irritation and corrosion on December 4th, 2014. The question is more appropriate for that webinar as this one discussed the skin irritation and corrosion endpoints. More info on that webinar can be found at this link: http://chemicalwatch.com/peta-webinars.

42. Is EpiOcular accepted for eye irritation testing by regulatory agencies at this stage?

    GEC: A draft OECD Test Guideline for the EpiOcular EIT is currently under review (see http://www.oecd.org/chemicalsafety/testing/RhCE-Test-Method-for-Identifying-Chemicals-Not-Requiring%20Classification-Labelling-for-Eye-Irritation-Draft-New-TG-2014-07-25.pdf). Given that the method has met validation acceptance criteria, it may be used for REACH purposes.