Questions and Answers from the CRACK IT Challenge 41 – SAFE launch webinar

1. **Are studies using invertebrates, such as *Drosophila* or *Daphnia, in* scope?**
   Invertebrate organisms, e.g., *Drosophila and Daphnia,* immortalized cell lines or unicellular organisms are in scope. Vertebrates, independent of their life stage (e.g., larvae and embryos), are out of scope for this Challenge.

2. **"Uncertainty analysis" is mentioned in the Challenge brief, could the Sponsors provide more detail about the objective of uncertainty analyses?**
   In the context of this Challenge the uncertainty analysis should aim to understand and if possible, quantitatively characterise the margin of error associated with the established assay. It should answer questions such as:
   a. Can the assay reliably predict the concentration of a compound causing an adverse effect with an error less than one order of magnitude?
   b. What is the false positive and false negative rate in case of a binary outcome when compared to well characterised compounds from the literature?

   However, this analysis should extend beyond statistics and aim to address questions regarding the possibility for inter species extrapolation (e g. transferability of the results between different model fish species) and applicability to different chemistry (e g. mode of actions, chemicals classes etc.). The exact scope of the analysis will depend on the nature of the assay and should be discussed with the Sponsors early in the Challenge process.

3. ***In silico* models often need experimental data to be validated and/or calibrated to assess the predictivity of the model. Is generation of *in vivo* data to do this acceptable?**
   It is not within the scope of the Challenge to perform *in vivo* studies to validate any models developed. All models should be validated using historical *in vivo* data available. Many *in vivo* data are publicly available. In addition, the Sponsors may provide historical non-published safety data for validation or calibration purposes, if needed and subject to agreements.

4. **When referring to adverse outcome pathways (AOPs) to inform bioassays/biomarkers, are Sponsors only considering those that are currently available in the literature or other resources (e.g., AOP wiki), or would plausible AOPs derived from existing knowledge about causality between mode of action and adversity from different stressors that are not currently in the literature be considered?**
   Either existing AOP or other plausible linkages that can be justified are acceptable - however it is important that the evidence of causality between the identified adverse effect and underpinning mechanism will be presented.
   Phase 1 of the Challenge aims to investigate what the unknown space in AOP prediction is. There may be relevant Adverse Outcomes (AO) which have not been mapped into an AOP but still fall under the scope of this Challenge which are not currently in the literature. The Sponsors are interested in proposals that go beyond knowledge that is already available to gain a more complete understanding of fish specific AO. This could be determined from either a bottom up (starting with a relevant molecular initiating event) or top down (starting with a relevant AO) approach.

5. **If permanent cells cannot yet be routinely cultured without serum, is it acceptable to use serum to establish bioassays?**
   If it has been shown that the selected cells cannot be cultured in serum-free conditions, the use of animal serum in culture media is permissible for use based on the assumption that they are 'off-the-shelf animal-derived laboratory reagents' and are generally available from commercial suppliers. There must be a justification of the chosen cell culture practice and detailed descriptions of alternatives that have been explored. We encourage considering whether there are any appropriate alternatives from a

non-animal source and plans to move to serum-free conditions in Phase 2 are recommended where possible.

6. **Is the generation of new cell lines (e.g., pluripotent stem cells) from adult fish tissues and/or embryos in scope?**
   The scope of this Challenge does not include the culling of vertebrate species to generate new cell lines.

7. **Are the Sponsors prioritising specific species such as tuna?**
   It should be highlighted that the objective of this Challenge is to reduce/eliminate the need for the use of animals in testing for regulatory safety purposes and not focusing on the protection of ecological or economical important species. For this, the Sponsors are not prioritising any specific species. The Challenge aims for new bioassays that would provide a point of departure to inform safety assessments / environmental risk assessment practices. Under this framework, freshwater species or appropriate substitute model species are usually considered. The Sponsors would also welcome innovative ideas such as unicellular models or *in silico* approaches.

8. **Do the assays need to meet any regulatory requirements?**
   Compliance with current regulatory requirements is not an essential condition, although the Challenge does highlight that the applicants and Sponsors will work towards ultimate regulatory acceptance of the developed bioassays. Thus, preliminary consideration of how the proposed assays align to current regulatory frameworks and what additional strategic steps may be needed to make them acceptable should be presented.