Animal-free *in vitro*: Replacement of animal-derived reagents in an established human cell-based *in vitro* assay associated with an OECD Test Guideline

Overall aim

The aim of the Challenge is to adapt established Organisation for Economic Development (OECD) Test Guideline (TG) *in vitro* assays so that they are free from animal-derived products, delivering a robust, human-relevant (and preferably chemically-defined) version of the assays that demonstrates improved data quality and reproducibility.

Duration

Phase 1: six months, Phase 2: Up to three years.

Budget

Phase 1: Up to £100k, Phase 2: Up to £0.6 million

Sponsors

Unilever and AstraZeneca

Background

The OECD Guidelines for the Testing of Chemicals are the international standard for assessing the potential effects of chemicals on human health and the environment. The Guidelines are regularly expanded and updated to ensure they reflect the state-of-the-art science and techniques to meet member countries regulatory needs. A growing number of *in vitro* assays are included in the Guidelines that can be used as alternatives to animal studies (European Commission, Joint Research Centre 2019). There is increasing interest for these assays to also be free from animal-derived products to improve human relevance and reproducibility, and to reduce the use of animals (Gstraunthaler *et al.,* 2013). This was highlighted during a joint NC3Rs/Unilever workshop 'Towards the development of animal product free *in vitro* systems' that was held in January 2020.

Animal-derived serum, especially foetal bovine serum (FBS), is widely used in *in vitro* assays as a supplement in cell culture media to support cell growth, but its production has animal welfare concerns and its use impacts on the reproducibility of experiments due to inherent batch-to-batch variation (van der Valk *et al.,* 2018). The complete composition of FBS is not known. The presence of animal-derived serum can alter the biological response of some human cell types causing further

issues with the relevance of the data generated (Shahdadfar *et al.,* 2005; Usta *et al.,* 2014). Use of human serum has been successful in some assays (Belot *et al.,* 2017), but its use in standard laboratory testing environments is limited by ethical concerns and availability (Jacobs *et al.,* 2019), driving the need for the development of chemically-defined media for use in *in vitro* assays.

It is increasingly recognised that knowledge of all the constituents of the cell culture medium used and their influence on cellular processes are important for improved experimental reproducibility (Baker, 2016; Hirsch and Schildknecht, 2019). Chemically-defined media is completely free from serum (both animal and human-derived) and all the chemical components and concentrations are known, eliminating any ethical and scientific quality issues resulting from the use of animal or human-derived components. However, while there have been successful studies using chemically-defined media (Usta *et al.,* 2014), it is technically very challenging, for example, to ensure it includes all the required components for a cell type (van der Valk *et al.,* 2010 and van der Valk *et al.,* 2018).

Other components used in *in vitro* assays that are animal-derived also provide scientific and welfare concerns. Sources of metabolism include the use of liver fractions from the rat (S9 fraction). While these are cheap, readily available and provide a sustained source of metabolic activity, the complete composition of these fractions is not known and there are significant differences in liver metabolism between rodents and humans. For example, humans differ from rodents in isoform composition, expression and catalytic activities of enzymes involved in drug metabolism, including Cytochrome P450s (Williams, 1974), reducing the reliability of extrapolating metabolism data from animal models to humans.

The majority of antibodies used in *in vitro* assays are generated using animals. Animal-derived antibodies have significant drawbacks, they are expensive and unstable, have variable specificity and can suffer from batch-to-batch variation, adversely affecting the reproducibility of research (Bradbury and Plückthun, 2015). Non-animal-derived antibodies and affinity reagents are mature technologies that are commercially available, amenable to most research applications and can offer significant scientific benefits (Gray *et al.,* 2016). This was highlighted in a recent report from the EURL ECVAM Scientific Advisory Committee[1], that concluded non-animal-derived antibodies can replace animal-derived antibodies in most laboratory applications (Viegas Barroso *et al*., 2020). Uptake of non-animal-derived antibodies however has been slow with issues such as initial costs and a lack of awareness within the scientific community acting as barriers to their wide uptake (Groff *et al*., 2020).

In this Challenge, an established *in vitro* assay with an approved OECD TG, is required to be modified to remove animal-derived reagents whilst still generating scientifically robust and reproducible data. Using an established OECD TG provides the opportunity to compare the performance of the newly established assay with the current assay using the positive and negative controls and performance criteria outlined in the Guideline. The OECD TGs selected for animal-free modification are in the areas of genotoxicity and reproductive toxicity:

[1]European Commission's Joint Research Centre's Scientific EU Reference Laboratory for alternatives to animal testing (EURL ECVAM) Scientific Advisory Committee.

1. ***In Vitro* Mammalian Cell Micronucleus Test** OECD TG487: For this Guideline, the focus will be on the use of human-derived cells (human peripheral blood lymphocytes or a human cell line, for example, TK6 cells), development of culture conditions avoiding the use of animal-derived components and the replacement of animal-derived S9 fractions as a source of exogenous metabolism. Otherwise the assay must meet the requirements of the Guideline, allowing short and long-term treatment and metabolic activation of pro-mutagens. Performance assessment will ultimately rely on the output of the assay based on its performance with suitable positive and negative controls.

2. **Performance-Based Test Guideline for Stably Transfected Transactivation *In Vitro* Assays to Detect Estrogen Receptor Agonists and Antagonists** OECD TG455: For this guideline the focus will be on the use of human-derived cells expressing the human estrogen receptor (e.g. ER-CALUX, ERα-HeLa-9903, VM7Luc4E2) and development of culture conditions avoiding the use of animal-derived components (ideally focusing on chemically-defined medium). Performance assessment will ultimately rely on the output of the assay based on its performance with suitable positive and negative controls.

One, or preferably both TGs, should be addressed in this Challenge. These assays are used across industries including the fast-moving consumer goods and pharmaceutical industries which will help drive their broad uptake. In addition, the components will be transferable to other assay types, increasing the toolbox of animal-free assays in the future. For example, an alternative source of metabolic activation to the standard rat-derived S9 fraction will be valuable in a wide range of *in vitro* assays such as metabolic stability screening and intrinsic clearance and metabolism.

Successful completion of this Challenge will deliver an adapted protocol free from animal-derived products based on the original OECD TG that generates data of comparable or higher quality (due to being both more human-relevant and reproducible) and which can be transferred to other laboratories to facilitate industry uptake. Although acceptance of the animal free-protocols by OECD falls outside the scope of the Challenge, some early interaction with UK authorities, to ensure potential for progression towards the OECD work programme and future acceptance as an Annex to the current OECD TG, is an expected deliverable throughout Phase 2 of the Challenge.

3Rs benefits

A number of components of *in vitro* assays are currently derived from animal sources.

These include:

1. **Metabolic Enzymes:** The provision of sources of metabolism for *in vitro* assays are mostly obtained from rat livers (Hubbard *et al.,* 1985). Rats are dosed with a substance such as Aroclor or phenobarbital to induce increased levels of liver metabolism before being killed and an S9 fraction of their liver collected, which is then added to assays.

2. **Antibodies:** An estimated one million animals are used per year in the EU alone for antibody production (Viegas Barroso *et al.,* 2020). Antibodies generated for use in research are often made by inoculating animals with the protein of interest along with an adjuvant to increase the immune response. Blood is then collected from the animals and their antibodies generated in response to the protein of interest isolated. Production typically involves the immunisation of between up to three animals (usually mice or rabbits, but sometimes sheep, chickens, goats or donkeys) per target. Additionally, multiple attempts are often needed to generate antibodies for certain targets, further increasing animal use.

3. **Serum:** Collection of serum, and specifically FBS, is a by-product of the dairy industry where blood is collected from the unborn unanaesthetised calf by a cardiac puncture. Recent estimates state approximately 600,000 litres of FBS are produced annually worldwide, corresponding to two million bovine foetuses (Brindley *et al.,* 2012 and van der Valk 2020).

Currently, assays carried out in OECD TG455 and TG487 use and/or contain a number of animal-derived products. If successful, this Challenge could lead to the acceptance of new protocol(s) in the OECD TGs that are free from animal-derived products, whilst also improving their human-relevance and robustness. More generally improved *in vitro* assays would be relevant to a range of industrial sectors including those where animal testing is not conducted (e.g. cosmetics) and those where *in vitro* assays are important screens prior to animal testing (e.g. pharmaceuticals).

Key deliverables

Applicants should select one, or preferably both, Guidelines to address during the Challenge.

Phase 1 deliverables

Provide evidence that progress has been made towards adapting the chosen cell line into animal-product-free conditions and provide a detailed plan for how the adapted version of the OECD TG will be performed if progressed to Phase 2, including a proposal for any other alternatives required (e.g. metabolic activation) where appropriate.

Guideline-specific considerations

The cell line chosen should be one that is human-derived. Ideally the new protocol will also be chemically-defined and not contain the use of human-derived products such as serum. This is

particularly relevant for OECD TG455, where treatment of serum with charcoal/dextran is currently necessary to remove endogenous hormones and hormone-binding proteins which, if present, can be a confounding factor in the interpretation of the results. However, if no suitable alternative is available, then use of human-derived products will be accepted.

1. **OECD TG487**: For this guideline, the focus will be on the:

    - Use of human-derived cells (human peripheral blood lymphocytes or a human cell line, for example, TK6 cells).
    - Development of culture conditions that avoid the use of animal-derived components and the replacement of animal-derived S9 fractions as a source of exogenous metabolism.
    - The assay should meet the requirements of the Guideline, allowing short and long – term treatment with the test chemical and metabolic activation of pro-mutagens.

2. **OECD TG455**: For this guideline the focus will be on the:

    - Use of human-derived cells expressing the human estrogen receptor (e.g. ER-CALUX, ERα-HeLa-9903, VM7Luc4E2).
    - Development of culture conditions avoiding the use of animal-derived components (ideally focusing on defined medium).
    - The assay should meet the requirements of the Guideline, allowing differentiation between weak and strong estrogen receptor agonists and antagonists.

Applicants should also include robust plans to deliver Phase 2 of the Challenge including for commercialisation and dissemination.

Phase 2 deliverables

Essential:

- Finalise the adaptation of the cell line into fully animal product-free cell culture conditions and demonstrate that growth – including morphology, viability, stability (including karyotypic stability essential for OECD TG487) of the cells – is equal or better than in the original cell culture conditions.
- Establishment of the adapted version of the OECD TG and clear demonstration that the data produced is of the same quality (or potentially higher due to being both more human-relevant, robust and reproducible) as the current TG assay. This should be done using the positive and negative controls, and performance criteria outlined in the OECD TG. However, any differences would be accepted if there are scientific reasons that a fully human-based assay would give different results for specific chemicals.

- Demonstration that the assay can be performed in at least one other laboratory (i.e. inter-laboratory comparison) and that the results are comparable.
- Publication of adapted protocol and results.

Desirable:

- Evidence of early interaction with UK authorities, and plans to ensure potential for progression of the revised method/guideline towards the OECD work programme (noting that acceptance of the animal-free protocols by OECD falls outside the scope of the Challenge).

Sponsor in-kind contributions

Since the 1980s Unilever scientists have been developing and applying a wide range of non-animal approaches (including the use of *in vitro* tests), so that the safety of Unilever products can be assured without the use of animal testing.

Unilever will provide:

- Expert input on use of the chosen OECD TGs and support over chemical choice for the assay evaluation.
- Sharing of expertise/knowledge of animal product alternatives.
- Access to academic and industry networks to facilitate industry uptake.

AstraZeneca will provide:

- Expert input on use of the chosen OECD TGs and support over chemical choice for the assay evaluation.
- Sharing of expertise/knowledge of animal product alternatives.
- Data from human and animal cell lines for comparison.
- In-house assessment of adapted assays and components.

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