Public Statement to accompany May 21, 2020 presentation at ICCVAM 2020 Public Forum

Choosing Modern Assay Technologies to Develop Test Guidelines

Although I have been involved with the commercial development of *in vitro* cytotoxicity assays for the last 30 years, I am not familiar with the process of formally validating methods to become one of the OECD Guidelines for testing of chemicals. As an outsider, it is not clear to me the rationale used for selection of which *in vitro* cell health assay options to include in methods that become validated or whether those choices are peer reviewed. In some cases, there are more scientifically and statistically sound assay methods that could have been used. I will point to two different examples that would have scientifically and statistically improved OECD guideline methods if modern assay options were chosen.

Example 1: OECD/OCDE Test No. 432 In Vitro 3T3 NRU Phototoxicity Test

The phototoxicity test uses mouse fibroblast cells with an endpoint of viable cell number measured using the multistep Neutral Red uptake assay.

Why choose rodent cells?
The rationale for choosing rodent cells to predict human phototoxicity is not clear. Given the possibility of species specific effects, a non-transformed human keratinocyte or a three dimensional dermal equivalent model may have been a more physiologically relevant choice to predict outcome of exposure to humans.

Why use Neutral Red Uptake?
The Neutral Red Uptake assay measures accumulation of a vital dye in lysosomes as a marker of cell viability. The assay protocol requires multiple medium removal and “gentle wash” steps which are not clearly defined in the OECD guideline document. In many cases, dead cells become detached from the plastic surface and are prone to removal upon washing steps. The greater the number of wash steps in the protocol (before and after addition of Neutral Red), the higher the likelihood of increased variability among replicate samples.

Alternative Approaches:
There are much simpler alternatives available for determining the number of viable cells present after chemical treatment. For example, the quantity of ATP is well established as a marker of viable cells. The ATP assay is more widely accepted and has become the gold standard for *in vitro* cytotoxicity testing, especially for high throughput screening labs. [qHTS assay for cell viability of HEK293 cells. PubChem AID: 743288 https://pubchem.ncbi.nlm.nih.gov/bioassay/743288]. The ATP assay is homogeneous, involving a single reagent addition followed by a brief mixing step using a plate shaker, then recording luminescence which is proportional to the quantity of ATP and viable cell number. It is by far the most sensitive and has fewer compounds that interfere with the assay chemistry compared to other methods.

There also are alternative *in vitro* assay options available to simultaneously measure both viable and dead cells from the same sample in real-time using a homogeneous method. Homogeneous means the protocol involves addition of reagent directly to the sample of cells with no medium removal or wash steps. The combination of measuring viable and dead cells serves as an internal control to confirm cytotoxicity results using orthogonal methods measuring different markers. [Hsieh J-H, Huang R, Lin J-A, Sedykh A, Zhao J, Tice RR, et al. (2017) Real-time cell toxicity profiling of Tox21 10K compounds reveals cytotoxicity dependent toxicity pathway linkage. PLoS ONE 12(5): e0177902. https://doi.org/10.1371/journal.pone.0181291] An increase in dead cells can be used to confirm a decrease in viable cell number. Recording kinetic data in real-time to monitor changes in both the viable and dead populations of cells is easily accomplished and more informative than using an individual endpoint assay such as Neutral Red uptake.

Example 2:
OECD/OCDE TG 442D In Vitro Skin Sensitisation: ARE-Nrf2 Luciferase Test Method

The KeratinoSens (and LuSens) In Vitro Skin Sensitisation assay uses a firefly luciferase reporter method to measure the expression of the ARE-Nrf2 gene. The protocol recommends using a parallel set of samples (in a separate assay plate) as a control to test the effects of treatments on the viability of the cells measured using an MTT assay. The protocol described for the MTT assay includes medium change steps, thus it is not homogeneous method. It also does not take into account that MTT itself is cytotoxic [Riss TL, Moravec RA, Niles AL, et al. Cell Viability Assays. 2013 May 1 [Updated 2016 Jul 1]. In: Sittampalam GS, Grossman A, Brimacombe K, et al., editors. Assay Guidance Manual [Internet]. Bethesda (MD): Eli Lilly & Company and the National Centerfor Advancing Translational Sciences; 2004- https://www.ncbi.nlm.nih.gov/books/NBK144065/pdf/Bookshelf_NBK144065.pdf].

Alternative Approachs:
There are assay options available for measuring viability markers directly in the same sample well as the KeratinoSens luciferase reporter assay rather than using replicate plates. For example, assay chemistries are available for fluorescent multiplex measurement of both live & dead cell markers from the same sample as that used to measure the firefly luciferase reporter (https://www.promega.com/-/media/files/resources/protocols/technical-bulletins/101/multitox-fluor-multiplex-cytotoxicity-assay-protocol.pdf?la=en). Multiplex measurement of the desired markers from the same sample using a multimode plate reader is more consistent and statistically more powerful than recording data from separate parallel culture plates.

Recommendation:
Scientists developing *in vitro* cell health assays that are proposed to become OECG guidelines should consider reaching out to the scientific staff of the vendors providing the assays to seek technical input regarding the choice of assays and potential for multiplexing. While individual vendors may not be in a position to formally validate *in vitro* assays for a particular regulatory purpose, in many cases, they have the most detailed understanding of the assay chemistries including the advantages, limitations, chemical interferences and the compatibility for multiplexing. There is often the assumption that anything presented from vendor scientists has the primary purpose of promoting sales of specific products; however, this is not always the case. Vendor representatives frequently participate in educational outreach training workshops sponsored by NCATS, presenting best practices described in the NIH Assay Guidance Manual (https://www.ncbi.nlm.nih.gov/books/NBK53196/). Educational workshops have been held at NCATS, the FDA, the Society for Laboratory Automation and Screening, the Society for Toxicology, the FDA, University of North Carolina at Chapel Hill, etc. and are available on-line (https://ncats.nih.gov/expertise/preclinical/agm/training#online-training). Relevant to the above examples, the most frequently accessed chapter in the Assay Guidance Manual is the one describing cell viability assay chemistries along with their advantages and limitations.

Thank you for the opportunity to comment.

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