How do I know that I know how? A systematic approach to implement a new assay

Marilyn Registre, Crystal Wylie, Marise Roy, Annie Hamel and Renato Cardoso
Charles River Laboratories Montreal ULC, 22022 TransCanada Highway, Senneville, Quebec, Canada, H9X 3R3

charles river

1 ABSTRACT

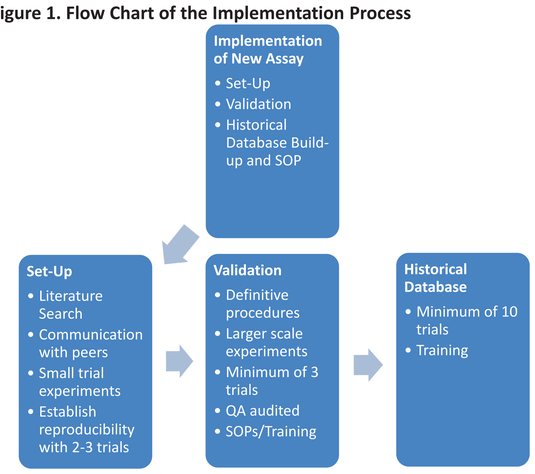
Genetic toxicology is an evolving field with constant development of new assays. When implementing a new assay, it is crucial to adopt checkpoints to certify that the assay is under control and performing adequately. With the last update of the OECD guideline 476, some recommendations were added for important parameters to consider when implementing *in vitro* mammalian cell gene mutation using the HPRT gene. At Charles River MTL, we adopted an approach to implementing new assays that incorporates aspects of the OECD recommendations and applied it to different genetic toxicology assays. The approach is divided in two phases: set up and validation. The set-up phase comprises a series of small non-GLP experiments, looking at optimizing individual aspects of the procedures. A selection of the initial experimental conditions to be tested is based on revision of the literature and consulting with groups that have experience with the assay. In this phase a number of genotoxic-positive substances acting via different mechanisms (at least one active with and one active without metabolic activation) and negative controls using solvents/vehicles are used. Typically, three independent experiments are performed with positive control substances investigated in the absence and in the presence of metabolic activation, in order to demonstrate proficiency to detect mutagenic substances, to determine the effectiveness of the metabolic activation system, and to determine cell viability. A range of concentrations of the selected substances is tested and should provide reproducible concentration-related increases in the variable measuring genotoxicity. If changes are needed to reach an adequate response, the experiment is repeated and the parameters are only selected for validation once reproducibility is demonstrated.

As the method is established and gives good quality results, consistent with those obtained in other reliable laboratories, then the method is validated under GLP conditions. Two concentrations of positive controls that provided a clear positive response (but not immediately obvious) are selected for validation. The validation includes multiple parameters such as intra-, inter-assay precision, day-to-day variability, sensitivity, stability assessment of samples (e.g. cell suspension, unfixed/fixed samples, long term storage of solutions, slides, etc.). Typically, 10 to 20 experiments are performed by different analysts. The historical data is built and 95% interval of confidence is determined. The standard protocols are then issued and technical and scientific staff are formally trained on the new method.

If the test is a variation of a method that is already established in the laboratory (*e.g.* validation of a different cell line), then only a limited cross-validation may be needed to confirm that results are consistent with the original method and a historical database is built prior to performing GLP projects.

2 METHODS

Figure 1. Flow Chart of the Implementation Process



TK6 cells, a p53-competent human lymphoblast cell line, were obtained from the American Type Culture Collection (ATCC). Approximately 24 h prior to treatment with the reference compounds, TK6 cell stock cultures were prepared in vented T-75 cm^2 flasks at a cell density of 1.5 × 10^5 cells/mL. Stock cultures were incubated at 37 ° C in 5% CO_2 in upright flasks for 22–26 h.

Prior to utilizing cells for dosing, the cell density of the stock cell suspension was adjusted to 3.0 × 10^5 cells/mL and 2.5 mL aliquots were dispensed into Nunc tubes (4 h pulse treatments) or 5 mL aliquots were dispensed into T-25 cm^2 tissue culture flasks (27 h continuous treatment). Appropriate cultures were exposed in the presence of a crude Aroclor-induced rat liver homogenate/cofactor mix to simulate mammalian metabolism. Appropriate concurrent vehicle controls were included for each treatment regime.

Table 1: Reference Compounds used

Category	Compound	CAS No.	Conc. tested (µg/mL) and exposure
1. Clastogen active without metabolic activation			
	Mitomycin C	50-07-7	- 0.063 and 0.125 (4-hour, both set-up and validation) - 0.03 and 0.06 (27-hour, set-up #1 only)
2. Clastogen requiring metabolic activation			
	Cyclosphophamide monohydrate	6055-19-2	- 4.7 and 11.9 (both set-ups), 20.0 and 30.0 (added in validation)
3. Aneugens			
	Vinblastine	143-67-9	- 0.005 and 0.006 (4-hour, set-up #1 only) - 0.0025 and 0.0030 (27-hour, both set-up and validation)

Following the 4 h treatments, supernatant was replaced with fresh complete medium and incubation was continued for a further 40 h prior to harvesting. The 27 h treatment cultures were harvested at the end of the treatment period. At harvest, an aliquot of each culture was removed for counting using the Coulter counter. These counts were used to calculate Population Doubling (PD), Relative Population Doubling (RPD) and cytotoxicity:

PD=[log(cell density^b/cell density^a)]log 2
RPD = (the number of population doublings in the treated cultures/the number of population doublings in the negative controls cultures) × 100
% Cytotoxicity=(100−RPD)
where *b* = current cell density and *a* = previous cell density.

Following hypotonic treatment (0.075M KCl) and fixation (9:1 v/v, methanol: acetic acid), cells were dropped onto clean slides and air-dried before staining with the fluorescent metachromatic dye, acridine orange. Slides were randomized then encoded to minimize potential operator bias. A total of 2000 mononucleated cells per experimental point (1000 per culture) were examined for the presence of micronuclei using oil-immersion optics.

3 RESULTS AND DISCUSSION

Set-up
Two set-up trials were performed. In the first one, MMC and VIN were tested in both the 4- and 27-hour exposures without metabolic activation, and CP in the 4-hour exposure with metabolic activation, with two concentrations tested for each reference compound. Based on the good genotoxic responses obtained for MMC and VIN in the 4- and 27-hour exposures, respectively, the second set-up trial was performed accordingly, *i.e.* opting for the better genotoxin in these regimes and dropping the other one. Based on the low cytotoxicity results obtained for CP in Set-up#2, two additional concentrations were added in the validation phase.

Validation
Table 2: Summary Results and Statistical Analysis for 3 trials of the GLP validation of the In Vitro Micronucleus Test using TK6 Cells

			Trial 1		Trial 2		Trial 3	
Exposure	Treatment	Conc. (µg/mL)	Cytotoxicity[a] (%)	MN1 (%)	Cytotoxicity[a] (%)	MN1 (%)	Cytotoxicity[a] (%)	MN1 (%)
4-hr -S9	Untreated	-	-6	0.3	-1	0.8	0	1.4
	DMSO	-	0	1.0	0	0.5	0	0.9
	MMC	0.065	16	5.2**	24	1.8**	27	3.5**
		0.125	41	4.8**	49	5.0**	31	3.7**
4-hr +S9	Untreated	-	-11	0.4	-1	1.4	-3	0.8
	DMSO	-	0	0.8	0	0.7	0	0.9
	CP	4.7	10	0.8	3	0.3	14	2.8**
		11.9	17	1.6*	29	2.2**	18	1.7*
		20.0	56	3.2**	54	3.8**	45	4.1**
		30.0	83	3.8**	51	5.3**	71	4.5**
27-hr -S9	Untreated	-	7	0.8	11	1.2	3	0.8
	DMSO	-	0	1.00	0	0.5	0	0.8
	VIN	0.0025	15	3.0**	26	3.2**	39	3.4**
		0.0030	23	7.9**	29	3.9**	42	6.6**

a = Relative to the vehicle control (DMSO).
mN_1 = Micronucleated mononucleated cells.
DMSO = Dimethylsulfoxide.
MMC = Mitomycin C.
CP = Cyclophosphamide.
VIN = Vinblastine.
*p ≤ 0.05, ** p ≤ 0.01 otherwise p > 0.05 Fisher's exact test with single-sided probabilities.

Cytotoxicity of MMC and VIN at the concentrations tested, did not exceed 50% in any of the three trials. Cytotoxicity of CP at 20.0 µg/mL was approximately 50% in all three trials and at 30.0 µg/mL was approximately at 50% (Trial 2) or exceeded 50% (Trial 1 and 3). All experimental points in all three trials were evaluated for micronuclei.
All genotoxic compounds tested induced a statistically significant increase in micronuclei compared to the concurrent vehicle control for at least one of the concentrations tested in all regimes. These responses were reproduced on 3 separate occasions (Trials 1 to 3).
MMC induced a positive result with concentrations 0.065 and 0.125 µg/mL. In the case of CP, a reproducible positive result was obtained with 11.9, 20.0, and 30.0 µg/mL. VIN led to a positive response with 0.0025 and 0.0030 µg/mL.

Historical Database
An additional 7 trials were conducted in order to total 10 trials for the building of an historical database prior to offering the service (the database has since been updated with the results of assays performed for sponsors). The negative controls included untreated cultures as well as cultures treated with DMSO.

Figure 2. Historical Database

These QA audited results were collected from non-GLP and GLP compliant *in vitro* micronucleus studies with TK6 cells performed from Jun 2017 to Aug 2017.

Negative Controls

Regime	% of micronucleated cells	SD	Upper 95% limit[1] (% of micronucleated cells)	Range	No. of Treatments
4-hour treatment without S9	0.93	0.37	1.65	0.3 - 1.5	20
4-hour treatment with S9	0.85	0.30	1.44	0.4 - 1.4	20
27-hour treatment without S9	0.80	0.22	1.23	0.5 - 1.3	20

SD = Standard deviation; 1 = Upper 95% limit is mean + 1.96 x SD.

Positive Controls

Positive Control and Regime	% of micronucleated cells	SD	Range	No. of Treatments
Mitomycin C (4-hour without S9)	3.33	1.23	1.8 - 4.6	10
Cyclophosphamide (4-hour with S9)	2.99	0.77	2.2 - 4.1	10
Vinblastine (long exposure without S9)	2.75	1.41	1.3 - 6.2	10

SD = Standard deviation

4 CONCLUSION

The Charles River MTL Genetox department systematic approach to implement a new assay was illustrated using the validation of the in vitro micronucleus assay in TK6 cells.

We gratefully acknowledge the excellent technical assistance of Sonia Thibault, Amélie Laforce, Louise-Hélène Grimard, Karen DiPerna, Karen Guerra and Stéphanie St-Jacques.

.