A High-throughput Analytical Framework for Efficient Analysis of In Vitro Micronucleus (MN) Dose-response Data
Results for 292 Substances

Byron Kuo[1], John Wills[1], Paul A. White[1], Francesco Marchetti[1], Andy Nong[1], Andrew Williams[1], Keith Houck[2], Raymond Tice[3], Carole L. Yauk[1]

[1]Environmental Health Science and Research Bureau, Environmental and Radiation Health Sciences Directorate, HECSB, Health Canada, Ottawa, ON,
[2]National Center for Computational Toxicology, Office of Research and Development, U.S. Environmental Protection Agency, Research Triangle Park, NC 27711, USA,
[3]Division of the National Toxicology Program, National Institute of Environmental Health Sciences, Research Triangle Park, NC 27709, USA

Abstract

Objectives: Health Canada assesses the health risks posed by chemicals in commerce in Canada. An important endpoint in chemical assessment is genetic toxicity (e.g., ability to damage DNA) which is associated with cancer and inherited genetic diseases. The *in vitro* micronucleus (MN) assay is a standard test used for genetic toxicology assessment. It measures genetic damage by detecting DNA fragments or extra chromosomes that are present outside the cell nucleus after cell division (i.e., micronuclei).

Method: In vitro MN frequency data for 292 chemicals was analyzed in collaboration with the United States Environmental Protection Agency (US EPA). Due to the number of the chemicals requiring assessment, a novel high-throughput analytical approach was developed and applied.

Results: A high-throughput analytical approach was developed including a robust decision tree and the use of benchmark dose modelling. The approach was then applied to classify test chemicals. Key elements of the decision tree include comparison of the observed DNA damage response to control groups, as well as scrutiny of relative cellular survival, and the nature of the dose-response. Using the developed paradigm, 157 (54%) of the chemicals were classified as positive for genetic toxicity, 30 (10%) as negative, and 105 (36%) as inconclusive.

Conclusions: While further evaluation and refinement of the approach is necessary, preliminary results suggest that a high-throughput version of the traditional MN assay, augmented by our proposed assessment approach, can improve the efficiency of genetic toxicity testing.

Relevance: This high-throughput analytical approach to analyze large in vitro MN assay data sets can provide a rapid screen for genotoxicity, particularly data-poor chemicals being evaluated under the Chemicals Management Plan.

Introduction

The *in vitro* micronucleus (MN) assay is a genotoxicity test that detects DNA fragments or extra chromosomes (micronuclei) in cells after cell divisions. DNA fragments and entire chromosome loss are caused by clastogenic and aneugenic mechanisms, respectively. The Organisation for Economic Cooperation and Development has a test guideline for standard genotoxicity assessment using this method has a test (OECD 2016). In this study, we obtained *in vitro* MN data sets from the US EPA and applied our extensive experience with this assay to produce an analytical pipeline to evaluate the potential genotoxicity of the agents tested.

Figure 1. A binucleated cell showing a micronucleus.

The data provided were for 292 chemicals in total (and their concurrent solvent controls). All agents were tested in Chinese hamster ovary (CHO) cells. Each chemical was tested under 19 concentrations (n=1 per concentration) alongside controls, in both the presence and absence of Aroclor 1254-induced, rat liver S9 for metabolic activation. Data quality was assessed by examining both positive and negative controls, in the presence and absence of S9.

Method: Micronucleus Assessment

We developed a decision tree approach to assess MN based on %MN (the percent of cells with MN) against batch-specific solvent controls, cytotoxicity and dose-response (Figure 2). Within this decision tree, cytotoxic refers to concentrations at which relative survival was < 40%; adequate refers to levels of cytotoxicity between 40-60% (a requirement in the OECD *in vitro* MN test guideline, unless the top concentration tested is 10 mM, 2 mg/mL or 2 µL/mL); non-cytotoxic refers to test concentrations with relative survival > 60%. Results were classified as 'Positive', 'Negative' or 'Inconclusive' for genotoxicity.

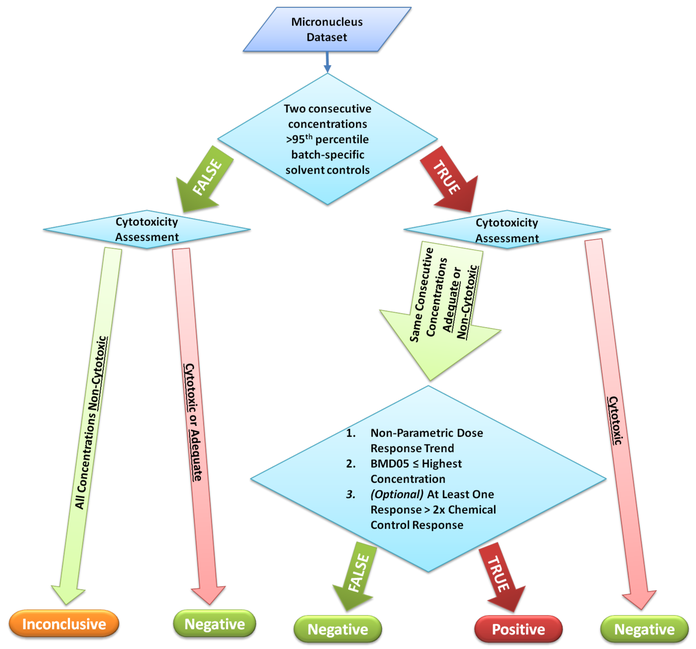

Figure 2. Decision making scheme for assessing MN datasetDecision making scheme for assessing MN data set

Benchmark Dose (BMD) Analysis

- For each chemical and S9 treatment, a %MN BMD value was computed based on 5% response (BMD05) using the PROAST package (version 61.2) for the R statistical environment.

Half-Maximal Activity (AC50)

- Half-maximal activity concentration (AC50) was computed for each MN positive chemical. AC50 was computed under the R statistics environment using the TCPL package (1.2.2) developed by the USEPA.

Method: Hypodiploidy Assessment

Hypodiploidy analysis detects changes in chromosome numbers, referred to as aneuploidy. Two approaches were evaluated for their ability to detect aneugenicity.

Approach #1: Applying the decision making scheme outlined above in the Micronucleus Assessment section, but replacing 95th percentile for MN with 95th percentile for batch-specific control hypodiploid percentages.

Approach #2: Applying method published by Bryce et al. (2011)

- Positive if fold change relative to concurrent controls in %MN is greater than three, and fold change for %hypodiploid cells is greater than ten.
- Negative otherwise

Results

We investigated the distribution of %MN and %Hypodiploidy of the solvent controls for each batch. Outliers were removed and batch-specific 95th percentile MN% and Hypo% were calculated for the decision-making scheme (Table 1).

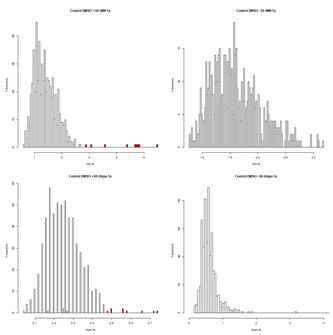

Figure 3. Overall distributions for negative control (DMSO) %MN and %hypodiploidy, with and without the addition of S9. For the %MN and %Hypo +S9 distributions, the nine outliers belong to 20131009 Plate 2 and are coloured in red. Four data points from Control DMSO –S9 (Hypo %) were removed (hypodiploid ranges from 15% to 132%).

Batch Date	MN 95th Percentile -S9	MN 95th Percentile +S9	Hypo 95th Percentile -S9	Hypo 95th Percentile +S9
20130827	1.86	1.22	0.84	0.30
20130829	1.80	1.75	0.64	0.38
20130903	2.31	1.81	0.56	0.32
20130905	1.92	2.00	0.61	0.42
20130906	2.31	2.29	0.81	0.37
20130909	2.47	2.24	0.66	0.39
20130911	2.96	2.10	1.46	0.36
20130916	2.65	2.06	0.76	0.37
20130918	2.47	2.24	0.82	0.44
20130923	2.28	1.99	0.80	0.41
20130925	2.21	2.31	0.77	0.38
20130930	2.36	2.12	1.36	0.49
20131007	2.19	2.12	1.03	0.44
20131009	2.46	2.00*	0.75	0.32*
20131015	1.65	1.61	0.93	0.54
20131017	1.84	1.36	0.74	0.32
20131024	2.28	2.00	0.70	0.41
20130916	2.65	2.06	0.76	0.37
20130918	2.47	2.24	0.82	0.44
20130923	2.28	1.99	0.80	0.41
20130925	2.21	2.31	0.77	0.38
20130930	2.36	2.12	1.36	0.49
20131007	2.19	2.12	1.03	0.44
20131009	2.46	2.00*	0.75	0.32*
20131015	1.65	1.61	0.93	0.54
20131017	1.84	1.36	0.74	0.32
20131024	2.28	2.00	0.70	0.41

Table 1. Upper 95th percentiles of the distribution of negative control (DMSO) %MN and %hypodiploidy (Hypo) were calculated based on the distributions of DMSO datasets per batch (date). *Data collected on "20131009 Plate 2" were removed from the calculation.

MN Assessment

Using the decision-making scheme outlined in Figure 2, 54% of the 292 chemicals were classified as MN positive, 10% negative and 36% inconclusive. We also applied an additional optional 2X fold change filter and identified 41% positive, 15% negative and 44% inconclusive (Table 2).

MN Assessment	Positive	Negative	Inconclusive
-S9	119 (80)	110 (149)	79 (79)
+S9	68 (64)	80 (84)	158 (158)
Total	**187 (144)**	**190 (233)**	**237 (237)**
# of Chemicals	**157 (121)**	**30 (43)**	**105 (128)**

Table 2. Results of MN assessments. Numbers in brackets are chemicals assessed with an additional filter of at least one response greater than two-fold of the chemical control (concentration of zero µL/mL).

Hypodiploidy Assessment

Approach #1 yielded 60% Hypodiploidy positives and 40% negatives, while Approach #2 yielded 5% positives and 95% negatives. Six of the 15 positive aneugens classified in Approach #2 were true positives based on published data.

Hypodiploidy Approach 1	Positive	Negative	Inconclusive
-S9	186 (151)	96 (131)	26 (26)
+S9	39 (34)	74 (79)	193 (193)
Total	**225 (185)**	**170 (210)**	**219 (219)**
# of Chemicals	**200 (167)**	**32 (39)**	**60 (86)**

Table 3. Approach #1 results of Hypodiploidy assessment using the same decision tree as shown in Figure 2, but replacing 95th percentile for MN with 95th percentile for hypodiploidy. Numbers in brackets are chemicals assessed with an additional filter of at least one response greater than two-fold of the chemical control (concentration of zero µL/mL).

Hypodiploidy Approach 2	Positive	Negative
-S9	13	295
+S9	5	301
Total	**18**	**596**
# of Chemicals	**15**	**277**

Table 4. Approach #2 results of Hypodiploidy assessment using the approach proposed by Bryce et al. (2011): positive if MN fold change above control is greater than three-fold and hypodiploidy fold change is greater than ten, negative otherwise.

Cross-Referencing Assessment Results

We obtained the analyses performed by the US Environmental Protection Agency (EPA) on the same data set. The EPA analyses only classified MN and hypodiploidy as either positive or negative (i.e., no inconclusives). In either the presence or the absence of S9, the EPA analysis classified 153 data sets (117 chemicals) as MN positive and 258 data sets (214 chemicals) as hypodiploidy positive. When we compared our MN positive classifications with that of the EPA, 97 (61.8%) and 85 (80.8%) chemicals were in common for the without and with two-fold change filter, respectively. For hypodiploidy, the numbers were 163 (81.5%) and 140 (83.8%), respectively (Figure 4).

We also obtained an analysis for genotoxicity potentials based on the Tox21 Phase 1 assay data (results kindly provided by Dr. Jui-Hua Hsieh). 66 of the 292 chemicals tested were positive in at least one of the five Tox21 genotoxicity assays. We cross-referenced our assessments of MN and hypodiploidy with these 66 Tox21 positives. The results showed that 45 and 44 of the 66 Tox21 positive chemicals were in the lists of MN positives and hypodiploidy positives, respectively (Figure 5).

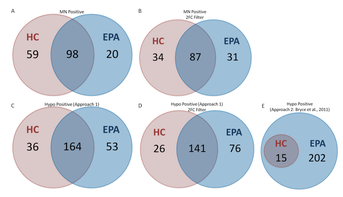

Figure 4. Comparison between HC and EPA MN and Hypodiploidy analyses without (A and C), with (B and E) a 2-fold filter cut-off, and between Bryce et al. (2011) and EPA Hypodiploidy analyses (E).

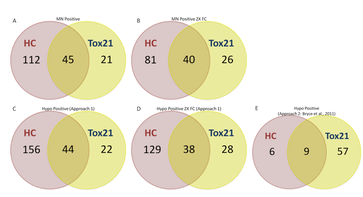

Figure 5. Comparison between HC and Tox21 genotoxicity assessment without (A and C), with (B and D) a 2-fold filter cut-off and between Bryce et al. (2011) and Tox21 genotoxicity assessment (E).

Conclusions

- We proposed a decision tree to assess high-throughput MN assay data sets for clastogenicity and aneugenicity. These results were compared with both EPA and Tox21 analyses.
- We recommend the two-fold filter on the %MN as we believe the data set may have a high number of false positives. We have confidence in the Bryce et al. approach for aneugenicity detection.
- Overall, the approaches and the results obtained from this analysis can be used to assign priorities for these substances based on potential genetox hazards for subsequent testing.
- The results of this analysis have been provided to the US EPA for considerations of screening high-throughput MN data sets.

Disclaimer

This poster does not reflect EPA policy.

References

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