IN VITRO MICRONUCLEUS (MN) ASSAY USING TK6 CELLS: REVIEW OF HISTORICAL POSITIVE AND NEGATIVE CONTROL DATA

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1 ABSTRACT

TK6 human lymphoblastoid cells are widely used in the in vitro MN assay as a part of regulatory genotoxicity testing for pharmaceuticals, but have seen relatively limited use in testing of other test materials, including tobacco-related products. As an established cell line, they do not exhibit the donor to donor variability observed in primary cells such as human lymphocytes. Unlike other routinely used p53-deficient cells (CHO, V79 and L5178Y), TK6 cells are p53-proficient, capable of DNA repair and normal cell cycle regulation, and generally have lower spontaneous MN frequencies and a lower rate of false or misleading positive results. We have used TK6 cells extensively to test a variety of e-liquids and e-vapor condensates, and total particulate matter (TPM) from combusted cigarettes. Here we present historical positive and negative control data, compiled from these OECD TG487- and GLP-compliant studies. The average negative control values (%MN) for the three treatments ranged from 0.93-1.03 with comparable ranges between different vehicles (negative controls). The known positive controls (two clastogens and one aneugen) run concurrently gave consistent and robust responses in %MN induction and ranged from 6.36-10.53. The consistency of results is critical since OECD guidelines place emphasis on the use of historical controls in evaluating individual assay acceptability and the biological relevance of results, and demonstrate the utility of this test system for evaluating tobacco- and ENDS-related products.

2 INTRODUCTION

The *in vitro* micronucleus (MN) assay evaluates the ability of a test article to break chromosomes or interfere with normal segregation of chromosomes during cell division. At telophase, these chromosome fragments and/or whole chromosomes are not segregated to either daughter nucleus and form micronuclei in the cytoplasm. Thus, this test can detect clastogenic and aneugenic agents.

With recent draft regulatory guidance for electronic nicotine delivery systems (ENDS), genetic toxicology assays are increasingly being used to evaluate cigarette and e-vapor products. OECD TG487[1] allows the use of various cell lines, including TK6, for evaluation of MN induction. As an established cell line, TK6 cells do not exhibit the donor to donor variability observed in primary cells such as human lymphocytes. And unlike other routinely used p53-deficient cells (CHO, V79 and L5178Y), TK6 cells are p53-proficient, capable of DNA repair and normal cell cycle regulation, and generally have lower spontaneous MN frequencies and a lower rate of false or misleading positive results.[2] Here we present our historical control database for ~200 TK6 MN assays, compiled from January 2015 to April 2018, as per Hayashi, et al (2011).[3]

3 MATERIALS AND METHODS

Cells

TK6 cells were obtained from the Genetic Toxicology Laboratory at Pfizer, Inc. (Groton, CT) and grown in RPMI 1640 with 10% (v/v) heat-inactivated fetal bovine serum, 2 mM L-glutamine, 100 U/mL penicillin and 100 µg/mL streptomycin (complete culture medium; CCM) under standard conditions (~37 °C in a humidified atmosphere of ~5% CO_2 in air).

Exogenous Metabolic Activation (S9)

Aroclor™ 1254-induced rat liver homogenate (S9) was obtained from Molecular Toxicology, Inc. (Boone, NC). Treatments +S9 contained (final) 0.33% v/v S9 homogenate and 400 µg/mL NADPH.

Treatment

Cultures were established (on Day -1) in T-75 cm^2 flasks in 50 mL CCM at 1.3 – 1.8 x 10^5 cells/mL and incubated overnight, upright, under standard conditions. On Day 1, cultures were seeded at 3 x 10^5 cells/mL in 2.5 mL in 15-mL centrifuge tubes for the 4-hr treatments ±S9, or 3 x 10^5 cells/mL in 5 mL in vented T-25 cm^2 flasks for the 27-hr treatment –S9). All test and control articles were evaluated in duplicate. Vehicle controls included various organic or aqueous solvents (at 1 or 10%, v/v, respectively). Two concentrations of each positive control were evaluated concurrently: mitomycin C (MMC); vinblastine sulfate (VIN); and cyclophosphamide (CP) as indicated (Table 1). At the end of the 4-hr treatment, cultures were washed and resuspended in 10 mL in fresh CCM in T-25 cm^2 flasks (27-hr treatments remained in T-25 cm^2 flasks in 5 mL throughout). Cells were harvested at the end of the 27-hour treatment –S9 (27 –S9), or at 44 hours (i.e., after a 40-hour recovery) for the 4-hour treatments ±S9 (4/40 ±S9). Cytotoxicity was based upon relative population doubling. Two thousand acridine orange stained mononucleate cells were scored for MN.

Guideline/ Regulatory Compliance

All assays reported herein were performed in accordance with OECD TG 487[1] and ICH S2(R1),[4] and in compliance with the Good Laboratory Practice regulations of the US FDA.[5]

4 RESULTS AND DISCUSSION

Historical control data from ~200 independent OECD TG487- and GLP-compliant assays for each type of treatment performed during January 2015 – April 2018 are presented (Table 1). Summary statistics include n, average, standard deviation (SD), range and lower/upper 95% control limits (LCL/UCL) for all individual cultures, as well as for the average value for the duplicate cultures within an assay. Control charts, indicative of the stability and reproducibility of the test system, also are presented for the same vehicle control data (Figures 1-3; average of duplicate cultures; positive control X-bar charts not shown due to space constraints).

Table 1. TK6 MN Historical Control Data (January 2015 – April 2018)

Treatment	MN Frequency (%)					
	4/40 -S9		4/40 +S9		27 -S9	
	Individual Pooled Vehicle Controls	Average Vehicle Controls	Individual Pooled Vehicle Controls	Average Vehicle Controls	Individual Pooled Vehicle Controls	Average Vehicle Controls
n	392	196	406	203	396	198
average	0.95	0.95	0.93	0.93	0.86	0.86
SD	0.38	0.32	0.34	0.28	0.36	0.30
range	0.10-2.20	0.25-1.85	0.20-2.20	0.35-1.90	0.10-2.10	0.25-1.70
95% LCL	0.21	0.32	0.27	0.38	0.16	0.26
95% UCL	1.69	1.57	1.60	1.49	1.56	1.46
	0.0625 µg/mL MMC		4.70 µg/mL CP		2.50 ng/mL VIN	
n	8	4	14	7	54	27
average	5.28	5.28	4.51	4.51	10.7	10.7
SD	0.56	0.47	1.27	1.19	3.08	3.03
range	4.50-6.40	4.90-5.95	2.60-6.90	2.95-6.50	5.00-18.4	5.30-18.0
95% LCL	4.17	4.35	2.02	2.17	4.69	4.77
95% UCL	6.38	6.20	6.99	6.84	16.7	16.7
	0.125 µg/mL MMC		11.9 µg/mL CP		3.00 ng/mL VIN	
n	372	186	378	190	* 332	166
average	7.35	7.35	6.17	6.17	9.94	9.94
SD	2.30	2.21	1.87	1.80	2.76	2.66
range	0.50-16.3	0.53-15.7	1.80-12.3	1.85-12.1	2.20-18.2	3.15-17.1
95% LCL	2.84	3.01	2.51	2.65	4.54	4.72
95% UCL	11.9	11.7	9.83	9.69	15.3	15.2

*Includes two experiments with 2000 cells scored/single culture (replicate lost due to technical error).

Figure 1. X-Bar Chart: Pooled Vehicle Controls, 4/40 –S9

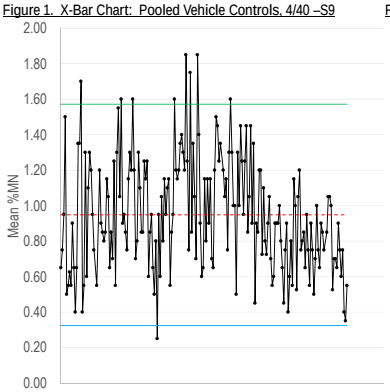


Figure 2. X-Bar Chart: Pooled Vehicle Controls, 4/40 +S9

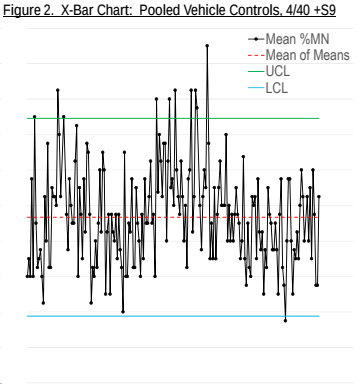
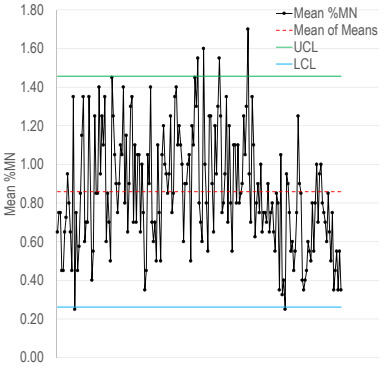


Figure 3. X-Bar Chart: Pooled Vehicle Controls, 27 –S9



For all control charts, the mean %MN values for duplicate cultures within an assay are presented chronologically for the period indicated.

5 REFERENCES

[1]OECD (2016) Test No. 487: In Vitro Mammalian Cell Micronucleus Test, OECD Guidelines for the Testing of Chemicals, Section 4, OECD Publishing, Paris.
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[5]United States Food and Drug Administration (1987) Good Laboratory Practice for Nonclinical Laboratory Studies, Title 21, CFR, Part 58.
[6]Sobol, Z. et al (2012) Development and validation of an in vitro micronucleus assay platform in TK6 cells, Mutation Research, 746:29-34.
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6 CONCLUSIONS

- Negative control data for each treatment type were consistent between assays; from the X-bar control charts, only 4.59, 5.42 and 3.03% of the values were outside the 95% upper and lower control limits (Figures 1, 2 and 3, respectively). Similar consistency in results was observed for the positive controls (not shown). However, there appeared to be a slight downward trend in vehicle control %MN across all three treatment groups at the end of the period.
- There were no differences in the negative control distributions ±S9 [one-way ANOVA ($F(2,1205) = 0.54$, $p = 0.58$), log transformed with offset]; inclusion of data generated after abstract submission reduced the variation between treatments even further (i.e., 0.86 – 0.95 now vs. 0.93 – 1.03 reported in the abstract; all values were updated with more recent results). These distributions and ranges compare favorably with published values.[6,7]