# Evaluation of artificial saliva as vehicle control in the *in vitro* micronucleus assay

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## Abstract

The *in vitro* micronucleus (MN) assay, one of the genotoxicity assays in the recommended battery of regulatory *in vitro* assays, routinely uses water, saline, or dimethyl sulfoxide as the solvent of choice. However, in the context of testing oral products containing nicotine, artificial saliva (AS) represents a more biologically relevant extraction solvent and hence would likely be the vehicle of choice for *in vitro* testing. In this study, we determined enzyme-free AS's effect as the vehicle control with the MN assay in human TK6 cells using three treatment conditions (short term ±S9 and long term -S9) as outlined in OECD TG 487. The objectives of the study were to 1) evaluate the effects of AS at different concentrations and identify the maximum acceptable concentration that can be used for *in vitro* genotoxicity testing of oral nicotine-containing products; and 2) monitor the baseline micronuclei frequency (% MN) using historical control data and X-bar charts at the selected dose(s) of AS. For the concentration-response study, TK6 cells were exposed to the vehicle control (complete culture medium, CCM) or varying concentrations of AS (1 – 50%, v/v, diluted in CCM), and cytotoxicity (measured as relative population doubling), osmolality, and pH were measured. AS up to 20% (v/v) did not elicit toxic effects and its pH and osmolality were within physiological range (260 – 320 mOsm), thus this concentration was considered the maximum acceptable concentration for vehicle control and was used for monitoring baseline frequency of % MN in the *in vitro* MN assay. Based on the historical results from 14 studies, the average % MN for AS in the three treatment conditions ranged from 0.40 – 0.45 and was in line with the published values. In summary, AS up to 20% (v/v) is appropriate to use as the vehicle control for oral nicotine-containing product testing in the TK6 based MN assay.

## Introduction

Smoke-free nicotine products, such as oral tobacco-derived nicotine (OTDN) products, are potential reduced-risk alternatives to conventional cigarettes for adult smokers who do not intend to quit. However, as no nicotine containing product is risk-free, these novel products are subject to comprehensive toxicological assessment for their cytotoxicity and genotoxicity potential *via* a battery of regulatory *in vitro* assays, including the micronucleus (MN) assay.[1,2]

All available methods propose the use of some type of extraction method using a solvent (organic or aqueous) to prepare an extract from oral products, which is then used to perform a toxicological evaluation. Artificial saliva (AS) has also been used as an extraction solvent as it represents a biological matrix more relevant to actual use patterns for oral-products testing purposes. However, considering that AS is not routinely used as a vehicle control in the MN assay, to support its use, further studies are required for the laboratory to establish a historical negative (solvent/vehicle) control range and distribution.

**Study Objectives**

1) Evaluate the genotoxicity (induction of micronuclei formation) of AS at various concentrations,
2) Select the maximum acceptable concentration that can be used for *in vitro* genotoxicity testing of oral nicotine-containing products,
3) Monitor the baseline micronuclei frequency (% MN) in AS against the lab's and literature historical control data with vehicle control.

## Materials and Methods

**Artificial Saliva (AS)**: The enzyme-free AS[3] was prepared and evaluated at concentrations of 1%, 5%, 10%, 15%, 20%, 25%, 30%, 35%, 40%, and 50% (v/v, diluted in culture medium).

***In vitro* micronucleus assay:** The human lymphoblast TK6 cultures were seeded at 2.5 to 3.5 x $10^5$ cells/mL in vented T-25 $cm^2$ flasks and exposed to the vehicle control (media) or the ten AS target concentrations under three different conditions: long-term incubation in the absence of activation (27h-S9) and short-term incubations in the presence and absence of metabolic activation (4h±S9). S9 mix was prepared from Aroclor™ 1254-induced rat liver homogenate or phenobarbital/5,6-benzoflavone-treated male rats. After treatment, the vehicle control, positive controls, and AS were assessed for the potential of inducing micronuclei, according to OECD TG 487 (OECD, 2016)[4] using *In Vitro* MicroFlow™ Kit (Litron Laboratories, Rochester, NY, USA). The MN frequencies of minimum 20,000 cells per treatment were acquired by flow cytometry and analyzed according to the manufacturer's instructions.

**Statistical Analysis and Evaluation Criteria:** The z' statistic was applied on the mean percentage of micronucleated cells to evaluate statistical significance between the test article treated groups and the concurrent vehicle control group, and the Cochran-Armitage test was conducted to assess dose-response trends.

- Assay acceptability criteria: i) the positive control results for each treatment condition must be statistically significantly different (z' ≥ 0.6) when compared with the relevant vehicle controls, and ii) the vehicle and positive control results should be comparable to the relevant historical control data generated at the CRO.
- Criteria for positive response: all of the following criteria should be met, in any of the experimental conditions examined i) at least one of the test concentrations exhibits a statistically significant (z' ≥ 0.6) increase in micronuclei compared with concurrent control, ii) the increase is dose-related in at least one experimental condition when evaluated with an appropriate trend test (Cochran-Armitage test, $p \leq 0.05$), and iii) any of the results are outside the distribution of the historical negative control data.
- Criteria for negative response: none of the above criteria are met.

The AS preparation was performed at Enthalpy Analytical, and the *in vitro* micronucleus assays were conducted at Charles River Laboratories.

## References

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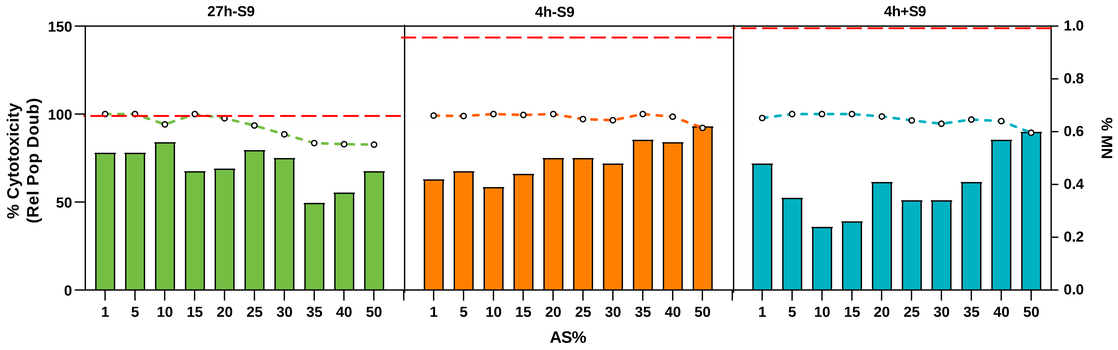
## Results

**Figure 1. Micronucleus concentration-response study.** Cytotoxicity (Relative Population Doubling) and genotoxicity (% MN) response following exposure to AS (concentrations varying from 1 – 50%, v/v) under 3 treatment conditions. The toxicity is represented by the curve, and the % MN responses are shown as bar graphs. The red dotted lines represent 95% CI-upper limit for vehicle control.




**AS did not elicit cytotoxic or genotoxic response up to 50% (v/v); however, based on osmolality values, 20% (v/v) was selected for further *in vitro* MN assays and baseline % MN responses monitoring.**

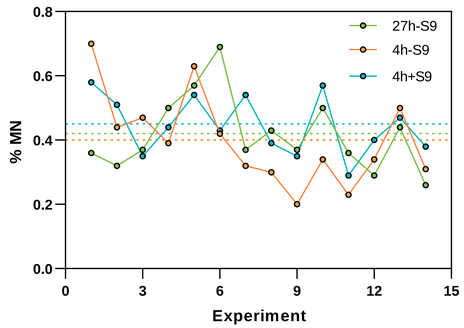



**Figure 3. X-bar chart indicating the study-to-study variability of the AS as vehicle control.** The dotted lines represent the mean counts of micronucleated cells from 14 independent experiments corresponding to each treatment type.

**The average %MN for AS in the three treatment conditions ranged from 0.40 – 0.45 and was in line with the published values* (0.10 – 2.20)[5,6] and CRO internal values* (0 – 0.99).**

*values corresponding to all the solvents used in these assays (water, saline, dimethyl sulfoxide, or AS)

**Figure 2. pH and osmolality of AS at varying concentrations (1 - 50%, v/v, diluted in culture medium).**

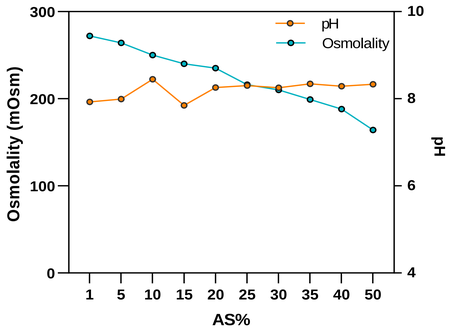



**Table 1. Summary of historical data of vehicle control-AS, from 14 independent OECD TG487- and GLP-compliant assays for each treatment type.**

| | MN (%) | | |
|---|---|---|---|
| **Treatment** | **27h-S9** | **4h-S9** | **4h+S9** |
| Average | 0.42 | 0.40 | 0.45 |
| Standard deviation | 0.12 | 0.14 | 0.09 |
| Range | 0.26 – 0.69 | 0.20 – 0.70 | 0.29 – 0.58 |
| 95% LCL | 0.18 | 0.12 | 0.26 |
| 95% UCL | 0.65 | 0.68 | 0.63 |

LCL – lower 95% confidence control limits
UCL – upper 95% confidence control limits

There are various vehicles available for *in vitro* micronucleus (MN) assays. For oral product testing, artificial saliva (AS) is physiologically relevant, and we demonstrate in this study that AS up to 20% (v/v) is acceptable as the vehicle control in the TK6 based MN assay.

## Conclusions

- This study shows that AS up to 20% (v/v) did not elicit toxic effects in the TK6 based MN assay and it is appropriate to use as the vehicle control for oral nicotine-containing product testing, as an alternative to the common solvents of choice (water, saline, and dimethyl sulfoxide).