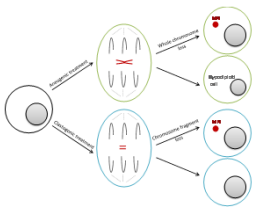
Performance of the flow cytometry-based *in vitro* micronucleus assay in a validation study

D.J. Smart, F.R. Helbling, B. Pouleau, D. McHugh and P. Vanscheeuwijck
Philip Morris International R&D, Philip Morris Products S.A., 2000 Neuchâtel, Switzerland
(part of Philip Morris International group of companies)

Introduction and Goal

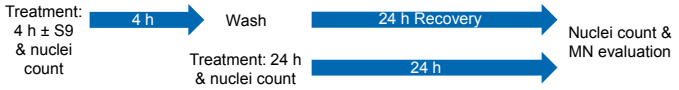
- The *in vitro* micronucleus (MNvit) assay is used to assess the genotoxic potential of a test substance by measuring the induction of micronuclei in cultured mammalian cells.
- The flow cytometry-based assay coupled with a high-throughput sampler permits the analysis of thousands of nuclei over a short period of time unlike the conventional slide-based assay.
- Furthermore, this version of the assay can discriminate the two major mechanisms by which MN may be induced:
  - Clastogenesis (chromosome breakage).
  - Aneugenesis (chromosome loss).



- The goal of this study was to evaluate the proficiency and variability of the assay. To this end, several reference chemicals as well as the total particulate matter (TPM) fraction generated from 3R4F research cigarettes were evaluated under 4 h ± S9 and 24 h treatment conditions.
- Proficiency: how accurately does the assay predict the genotoxic potential (and mechanism) of reference genotoxins and non-genotoxins?
- Variability: how variable are 3R4F TPM-induced responses over ten independent assays as well as within the same day? Furthermore, how variable are solvent-treated and positive control data?

Materials and Methods

- **Cell line:** Chinese hamster ovary-Wolff Bloom Litton (CHO-WBL; Merck Research Laboratories, USA). Cells were seeded in 96-well plates (Nunclon® microplates, NUNC) 24 h prior to treatment in McCoy's 5A + GlutaMAX™ medium + 10% Hi FBS + 100 units/ml Penicilin + 0.1 mg/ml Streptomycin and incubated at 37 +2/-3 °C, 5 ± 0.2% CO_2 + 70 ± 5 % humidity.
- **Aerosol generation:** 3R4F research cigarettes were conditioned for at least 48 h at 22 ± 1°C and 60 ± 3% relative humidity (ISO standard 3402) prior to being used for TPM generation. Following conditioning, TPM was generated via the Health Canada Intensive regimen using an RMB20 smoking machine (Burghart, Germany).
- **Testing procedure:**



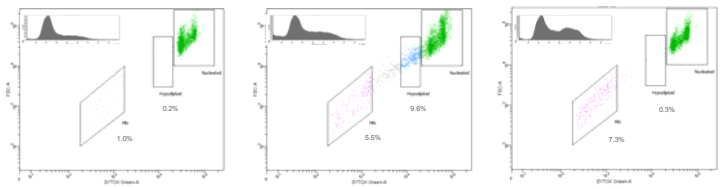
- **Genotoxicity assessment:** nuclei were prepared using the *in vitro* MicroFlow® kit (Litron Laboratories, USA) and analysed with a LSRII flow cytometer with an integrated high-throughput sampler (Becton Dickinson, USA). Concomitent absolute nuclei counts were made at the start and end of treatment (24 h) or end of the treatment-recovery period (4 h ±S9) and used to calculate the relative population doubling (RPD) cytotoxicity parameter. 5000 nuclei were evaluated in each assay.

Mechanistic Signatures

Non-Genotoxic Profile:
24 h: 1% v/v DMSO

Aneugenic Signature:
24 h: 0.07 µg/ml colchicine (COL)

Clastogenic Signature:
24 h: 20 µg/ml methyl methanesulfonate (MMS)



Response Characteristics	
Increase in MN	Increase in MN
Increase in hypodiploid nuclei	Negligible effect on hypodiploid nuclei
Non-specific cell cycle arrest	G_2M cell cycle arrest

Evaluation Criteria

- **Acceptability limits:**
  - ≥1.2 Population doublings in solvent-treated controls.
  - ≤2.0 %MN in solvent-treated controls.
  - ≥40% RPD in treated cells.
- **Genotoxicity evaluation:**
  - Positive response: Statistically signficant increase in %MN, positive trend and induced %MN outside the laboratory's ±2SD historical range (OECD Test Guideline 487).
  - Aneugenic mechanism: ≥10-fold increase in hypodiploid nuclei.

Responses to Reference Chemicals

Figure ①

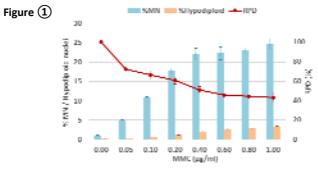


Figure ②

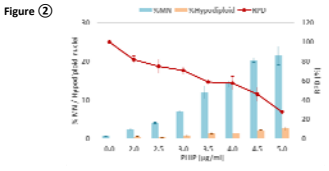


Figure ③

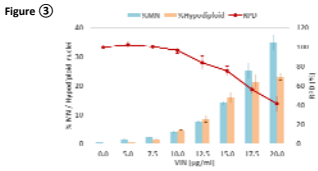
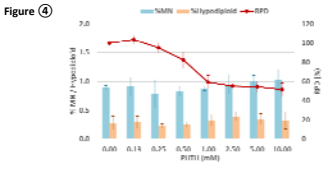


Figure ④



Examples of reference chemical-induced MN, hypodiploid nuclei and cytotoxicity:

Figure ① Treatment with the direct-acting genotoxin mitomycin C (MMC) for 24 h (n=3).

Figure ② Treatment with the pro-genotoxin 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine (PHIP) for 4 h with S9 metabolic activation + 24 h recovery (n=3).

Figure ③ Treatment with the aneugen vinblastine (VIN) for 24 h (n=3).

Figure ④ Treatment with the non-genotoxic cytotoxin phenanthrene (PHTH) for 24 h (n=3).

3R4F TPM (µg/ml)-Induced Responses

Figure ⑤

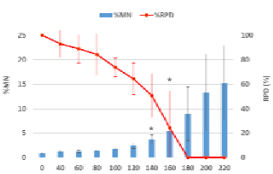


Figure ⑥

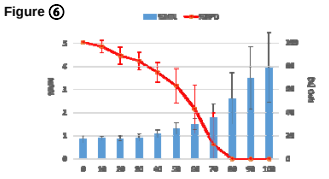


Figure ⑦

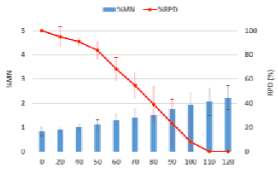
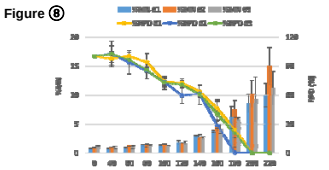


Figure ⑧



Figures ⑤ ⑥ ⑦ Mean concentration-response in 4 h +S9, 4 h -S9 and 24 h treatment conditions, respectively (n=10) (*P<0.05 statistically significant from control).

Figure ⑧ Three concentration-responses induced by the same 3R4F TPM dose formulation on one test occasion in the 4 h +S9 treatment condition.

%MN Control Charts

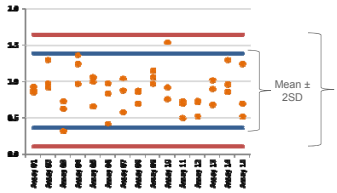


Figure ⑨ 24 h: Triplicate 1%v/v DMSO-treated controls

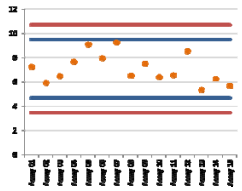


Figure ⑩ 24 h: 20 µg/ml Methyl methanesulfonate

Summary and Conclusion

- Known clastogens, e.g. MMC and PHIP, and aneugens, e.g. VIN, were found to induce marked genotoxicity as well as clear clastogenic and aneugenic mechanistic signatures, while non-genotoxic cytotoxins, e.g. PHTH, were confirmed as non-genotoxic in the assay.
- In the 4 h +S9 treatment condition, 3R4F-derived TPM induced consistent concentration-dependent increases in MN frequencies across the ten assays carried out with no evidence of aneugenicity. In the absence of S9, the responses were also consistent but less pronounced.
- Concentration-responses induced by the same 3R4F dose formulation on one test occasion were highly reproducible.
- Control charts reveal consistent background %MN as well as positive control-induced %MN.
- In conclusion, the flow cytometry-based MNvit assay detects genotoxic potential and mechanism of diverse test substances both accurately and consistently but also delivers stable levels of MN in control cell cultures, thus making the assay suitable for comparative genotoxicity assessments.

References

- Bryce S *et al.,* 2007 *Mutat. Res.* 630: 78-91; Bryce S *et al.,* 2011, *Environ. Mol. Mutagen.* 52: 280-286.

PMI RESEARCH & DEVELOPMENT

EUROTOX 2016
Seville, Spain
04-07 September

Philip Morris International Research & Development, Quai Jeanrenaud 5, 2000 Neuchâtel, Switzerland
T: +41 58 242 21 11, **F**: +41 58 242 28 11, **W**: www.philipmorrisinternational.com