

Miniaturization of the *In Vitro* Micronucleus Assay Using Human Peripheral Blood Lymphocytes: Application for Early Screening in Drug Development

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ABSTRACT

We have further miniaturized the *in vitro* micronucleus screen in isolated lymphocytes for use with minimal quantities of material by lowering incubation volumes to 250 μ L. Human lymphocytes stimulated with phytohemagglutinin for 48 hours were transferred to 96-well plates and incubated with vehicle, mitomycin C, cyclophosphamide, benzo(a)pyrene, and colchicine for 4 hours with an Aroclor™-induced rat liver S9 fraction and/or for 24 hours without metabolic activation. At the end of the incubation period, cells were washed, cytochalasin B added, and cells incubated an additional 18-20 hours. The cells were centrifuged onto glass slides, fixed with methanol, stained, and micronuclei scored from 400 binucleated cells per concentration. This screen was compared to our standard 10 mL whole blood human lymphocyte *in vitro* micronucleus assay. The micronucleus frequency using the microvolume screen was similar to that obtained from the 10 mL assay with mean %CV generally within 30% at a given test article concentration and condition. Cytotoxicity was measured using cytokinesis block proliferation index (CBPI) and tended to be higher for the microvolume versus the 10 mL assay but still within our 50-60% cytotoxicity target. The results of this microvolume screen indicate that it is suitable for evaluating drug candidates for genotoxic potential using minimal compound and is expected to be a reasonable predictor for the 10 mL volume *in vitro* micronucleus assay using human peripheral blood lymphocytes.

INTRODUCTION

Midwest BioResearch has been performing *in vitro* micronucleus screen in various cell types over the past 5 years and before that with the developers of novel *in vitro* micronuclei test systems at Monsanto/Searle/Pharmacia (Balwierz and Bunch 1998). The purpose of this validation study was to demonstrate that a low volume screen using HPBLs was as reliable as the standard full volume assay conducted using negative/positive controls as described in the draft OECD guideline.

METHODS

Microvolume screen: Blood was collected into heparinized tubes, lymphocytes isolated using density-gradient centrifugation and then stimulated with phytohemagglutinin (PHA-M) for 48-72 hours. 250 μ L cultures received vehicle (DMSO) at 1% or the positive control mitomycin C (MMC) or cyclophosphamide (CP), benzo(a)pyrene, and colchicine and incubated for 4 hours with an Aroclor™-induced rat liver S9 fraction or for 24 hours without an S9 fraction. Cytochalasin B was added at the end of these incubations, and cells incubated for an additional 18-20 hours, cytopun, fixed with straight methanol, stained, and micronuclei scored from at least 200 binucleate cells per slide.

10 mL volume assay: As above except whole blood cultured with PHA-M. At the end of the cytochalasin B exposure, cells were centrifuged, resuspended in hypotonic solution, fixed with methanol, added to slides, stained, and micronuclei scored from at least 200 binucleate cells per slide.

Cytotoxicity: For both methods, cytotoxicity was determined using the cytokinesis-block proliferation index (Kirsch-Volders et al.).

REFERENCES
Balwierz, P.S. and Bunch, R.T. Validation of an *In Vitro* Micronucleus Assay for Screening for Clastogenic/Aneugenic Activity. 1998 Environmental Mutagen Society Annual Meeting.

Organisation for Economic Cooperation and Development (OECD) Guideline for the Testing of Chemicals, Draft Proposal for a New Guideline 487: *In Vitro* Micronucleus Test (2nd Version), 21 December 2006.

Kirsch-Volders, M., Sofuni, T., Aardema, M. et al. Report from the *In Vitro* micronucleus assay working group. Mutat. Res., 540, 153-163, 2003.

RESULTS

Table 1. 250 μ L Cultures: Mean Summary

Concentration	Mean % MN BIS	SD (%)	MIN (%)	MAX (%)	Mean Cytotoxicity (% control)	N
24 Hours without S9						
DMSO (1%)	0.91	0.94	0.0	3.0	0	11
MMC (μg/mL)						
0.05	16	2.0	12	19	17	9
0.10	26	7.2	17	35	22	4
0.20	38	5.3	33	43	41	4
4 Hours with S9						
DMSO (1%)	0.30	0.27	0.0	0.50	0	6
CP (μg/mL)						
5	3.8	2.2	1.5	7.0	50	6
10	3.8	1.6	2.0	5.5	63	4
20	4.7	3.2	1.0	7.8	50	4

Table 2. 10 mL Cultures: Mean Summary

Concentration	Mean % MN BIS	SD (%)	MIN (%)	MAX (%)	Mean Cytotoxicity (% control)	N
24 Hours without S9						
DMSO (1%)	0.82	1.1	0.0	3.6	0.0	16
MMC (μg/mL)						
0.05	9.8	4.4	2.5	17	0.0	14
0.10	20	7.5	4.0	28	0.0	14
0.15	31	6.5	13	37	15	12
4 Hours with S9						
DMSO (1%)	0.52	0.59	0.0	2.0	0.0	20
CP (μg/mL)						
5	2.0	1.1	1.0	4.2	20	8
10	4.2	1.6	2.2	8.4	28	20
15	5.0	1.5	2.7	8.5	36	20
20	5.2	1.0	3.2	6.4	27	12

DMSO – Dimethylsulfoxide
MMC – Mitomycin C
CP – Cyclophosphamide
%MN BIS – Mean percent micronucleated cells scored per total number binucleate cells per slide
Cytotoxicity = $100 - 100[(CBPI_t - 1)/(CBPI_c - 1)]$
CBPI – Cytokinesis-block proliferation index
= $[(\#mononucleate\ cells) + 2(\#binucleate\ cells) + 3(\#cells\ with\ >2\ nuclei)]/total\ \#cells$

Figure 1.

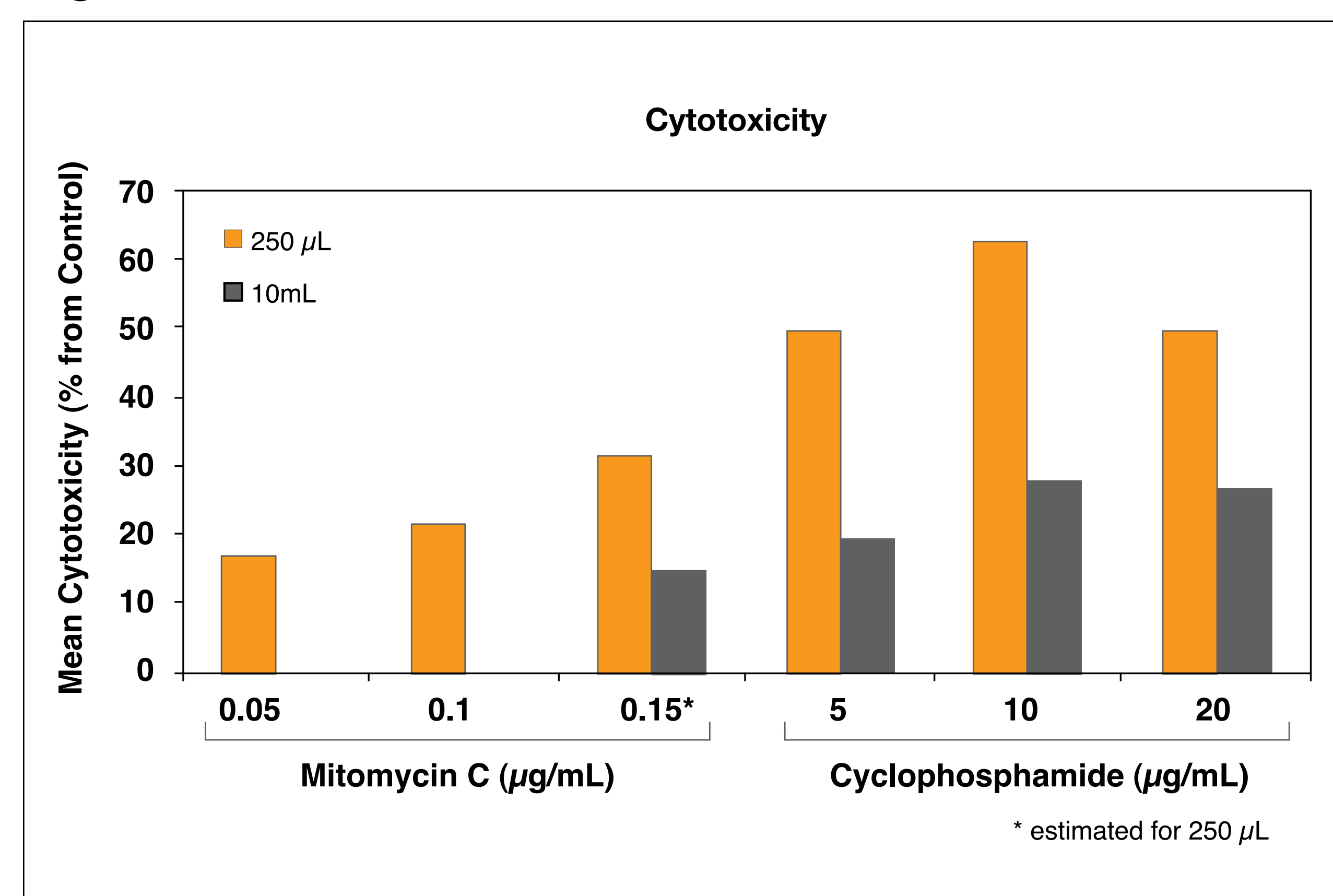


Figure 1. Comparison of cytotoxicity (i.e., cytokinesis-block proliferation index) for the microvolume screen (250 μ L) versus the standard format (10 mL).

Figure 2a.

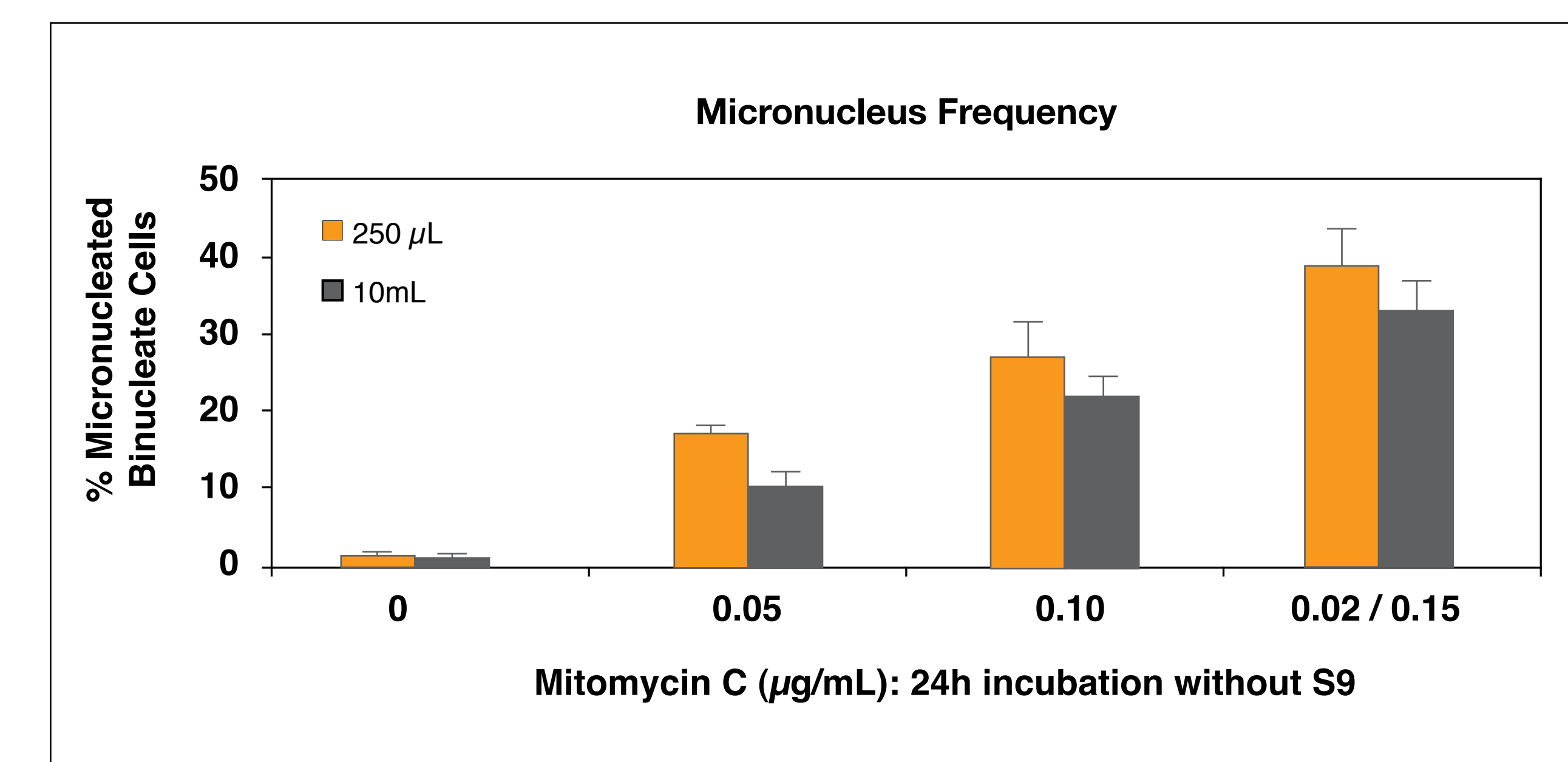


Figure 2a. Without metabolic activation: Comparison of percent micronucleus frequency (mean + standard error) in binucleate cells for microvolume screen (250 μ L) versus the standard format (10 mL).

Figure 2b.

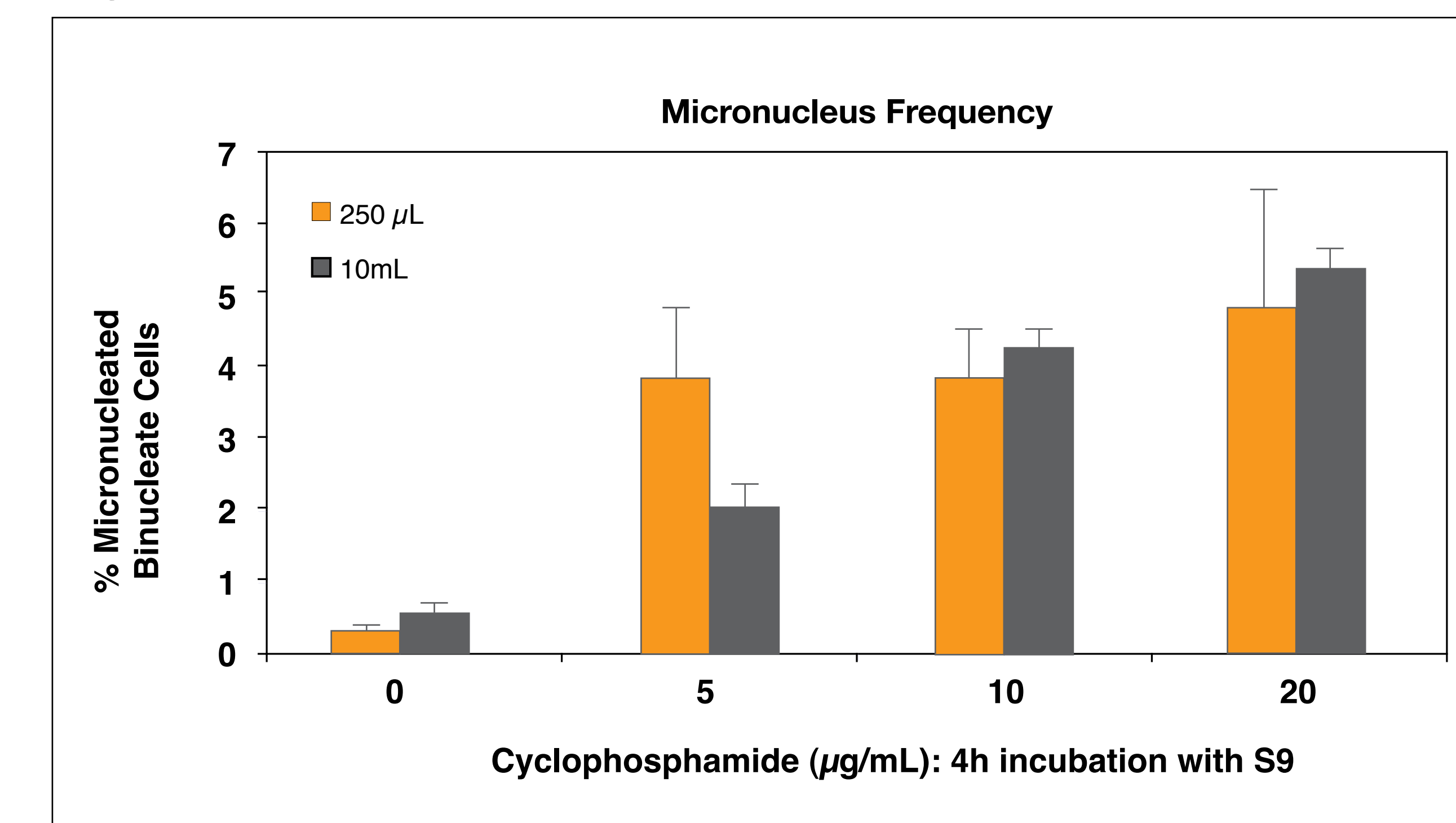


Figure 2b. With metabolic activation: Comparison of percent micronucleus frequency (mean + standard error) in binucleate cells for microvolume screen (250 μ L) versus the standard format (10 mL).

Table 3. 250 μ L Cultures: Mean Summary

24 Hours w/o S9	Conc. ng/mL	%MN BIS	Cytotox (% control)	4 Hours w/S9	Conc. μ g/mL	%MN BIS	Cytotox (% control)
DMSO	1%	0.50	0	DMSO	1%	0.50	0
Colchicine	4.19	0	61	B(a)p	10.2	0.50	41
	10.5	0.50	67		25.6	1.3	52
	26.2	2.4	78		64	3.0	62
	65.5	--	100		160	4.5	66

DMSO – Dimethylsulfoxide
B(a)p – Benzo(a)pyrene
%MN BIS – Mean percent micronucleated cells scored per total number binucleate cells per slide
Cytotoxicity = $100 - 100[(CBPI_t - 1)/(CBPI_c - 1)]$
CBPI – Cytokinesis-block proliferation index
= $[(\#mononucleate\ cells) + 2(\#binucleate\ cells) + 3(\#cells\ with\ >2\ nuclei)]/total\ \#cells$

CONCLUSIONS

The results of the 10 mL assay and 250 μ L screen were very similar for all treatment groups and conditions. Although cytotoxicity was generally higher in the microvolume screen versus the standard assay, the induction of micronuclei tended to also be greater for the microvolume screen. With both formats, cytotoxicity was typically within or below our 50-60% criteria. These data indicate that the microvolume screen is suitable for predicting micronuclei formed in the standard volume assay at early stages. This screen can be used at concentrations up to 5 mg/mL with only 10 mg and thus has application for screening compounds early in drug development when compound supply is limited.