

ABSTRACT

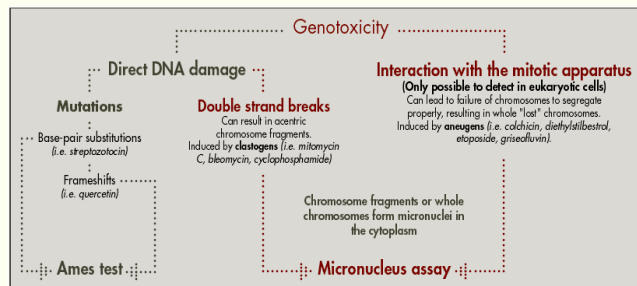
Approval and registration of drugs requires a comprehensive assessment of their genotoxic potential. In recent years, genetic toxicity testing has moved towards the earlier stages of drug discovery in order to identify genotoxicity liabilities as soon as possible. The *in-vitro* micronucleus assay is a widely used screening test which shows a high level of concordance with regulatory chromosomal aberration assays.

Traditionally, micronucleus detection is done through manual reading of slides. We describe the validation of an automated method for *in-vitro* micronucleus analysis, using 96-well plates and automated image analysis. The advantages of this method (small amount of compound required, rapid turnaround times, and objective/consistent scoring) make it ideal for early genotoxicity screening.

We show that this assay can accurately detect micronucleus induction by clastogens (mitomycin C, bleomycin) and aneugens (colchicines, diethylstilbestrol, etoposide, griseofulvine). We also describe the incorporation of a metabolic activation system that allows the detection of micronucleus induction by indirect clastogens such as cyclophosphamide.

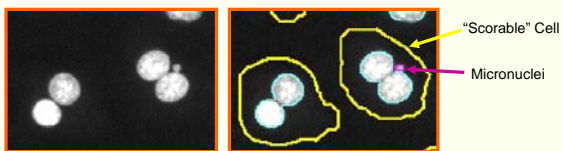
We also describe the incorporation of two cytotoxicity indexes to this assay: the cytokinesis-block proliferation index (CBPI) and total cell counts. These indexes complement each other and help in the interpretation of micronucleus data.

Figure 1: Types of genetic damage detected by the micronucleus assay



Genetic toxicity testing has moved towards the earlier stages of drug discovery in order to identify genotoxicity liabilities as soon as possible. Assays such as the mini-Ames test and the *in-vitro* micronucleus assay are routinely used for screening drug discovery compounds. The *in-vitro* micronucleus assay is commonly used as a pre-screening test for the regulatory *in-vitro* chromosomal aberration assay since it shows a high degree of concordance and it has many advantages over this assay (lower cost and faster turnaround time, less compound required, more statistical power and the ability to detect aneugens).

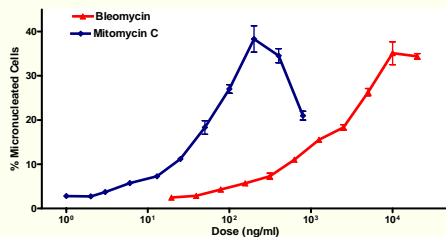
Figure 2: Automated scoring of micronuclei



The automated *in-vitro* MN assay is conducted in a very similar way to the standard manual *in-vitro* micronucleus assay, with the main difference being the **scoring of the cells**. The manual *in-vitro* MN assay uses trained operators to visually read slides under a microscope, and the automated assay uses proprietary image-analysis software designed by Cellomics (Pittsburg, PA) to score the cells. Detecting micronuclei in binucleated cells is a straightforward process that makes this assay an excellent candidate for image-analysis based automation.

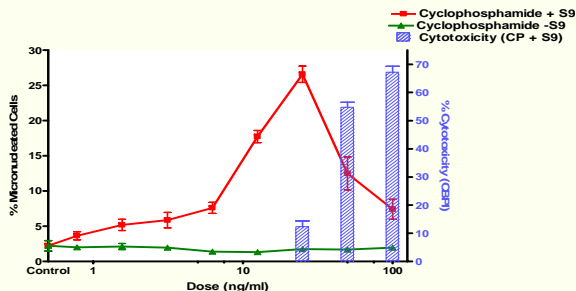
A "scorable" cell is a binucleated cell contained within the field boundary, in which both nuclei are of similar size and intensity. Micronuclei can be selected based on size (usually a diameter of < 0.33) and intensity relative to the main nuclei.

Figure 3: Micronuclei frequency induced by typical clastogens



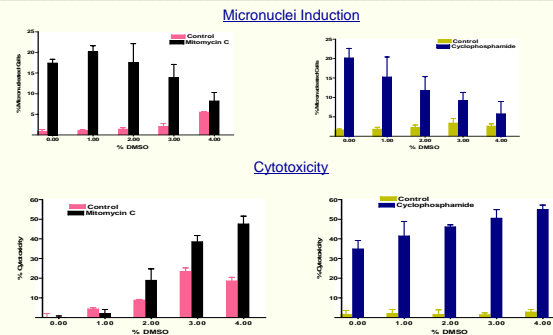
Typical direct clastogens like bleomycin and mitomycin C induce dose-response increases in micronuclei frequency. CHO-K1 cells were treated with chemicals for 24 h, after which cytokinesis was blocked with cytochalasin-B, and cells were incubated for another 24 h. Results are shown as the mean ± SD of 3 separate wells (~ 3,000 scored cells per concentration). The decrease in % micronucleated cells at high doses of mitomycin C is likely due to excessive cytotoxicity.

Figure 4: Micronuclei frequency induced by cyclophosphamide (indirect clastogen)



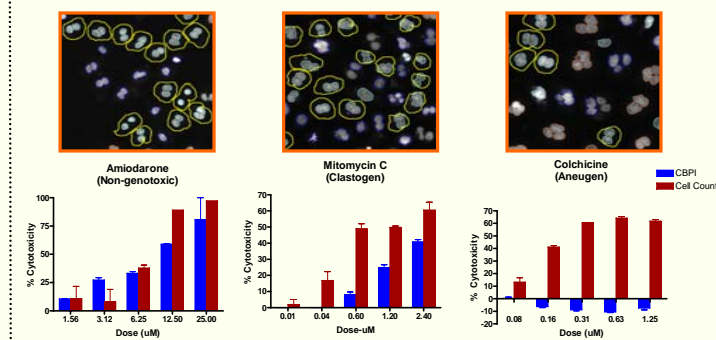
Indirect clastogens like cyclophosphamide induce a dose-response increase in micronuclei frequency only in the presence of metabolic activation (xx). CHO-K1 cells were treated with cyclophosphamide in the presence of rat liver S9 and a NADPH regeneration buffer for 3 h. The decrease in micronuclei at the highest concentrations is likely due to increased cytotoxicity.

Figure 5: DMSO tolerance in the in-vitro micronucleus assay



Increasing concentrations of DMSO have the following effects: increased background level of micronucleated cells after 24 h of exposure, decrease in the % of micronucleated cells induced by direct and indirect clastogens, increase in the cytotoxicity of direct and indirect clastogens. As a result, the DMSO tolerance level has been established at ≤ 1%.

Figure 5: Cytotoxicity in the in-vitro micronucleus assay



The assessment of cytotoxicity is done using two independent methods: proliferation index and total cell numbers. The first method measures cytotoxicity with a modified version of the cytokinesis-block proliferation index (CBPI) recommended in the OECD guidelines. This method takes advantage of the fact that cytotoxicity very often induces cell cycle arrest, which is reflected in a decreased ratio of binucleated to mononucleated cells when using cytochalasin-B. For most drugs, there is a good correlation between these two indexes (i.e. amlodaron and mitomycin C), however, for aneugens like colchicine the CBPI index is not a useful marker of cytotoxicity (aneugens increase the number of multinucleated cells), in which case cell numbers provide a useful indication of cell health.

Figure 6: Validation results with various chemicals

Chemical	Genotoxicity Classification	Micronucleus Formation		Max % cells with MN		Fold increase in MN		LOEL ^a uM	Dose-range Tested (uM)
		- S9	+ S9	- S9	+ S9	- S9	+ S9		
2-Aminofluorene	clastogen (indirect)	-	-	2%	2%	1.5X	0.9X	NA	35-552
Colchicine	aneugen	+	+	9%	15%	6x	6x	0.31	0.08-1.25
Cyclophosphamide	clastogen (indirect)	-	+	2%	27%	1x	12x	11.3	1.45-181
Diethylstilbestrol	aneugen	+	-	11%	2%	7x	0.9x	23.3	11.6-166
Etoposide	aneugen	+	+	22%	13%	14x	5x	0.09	0.02-0.34
Griseofulvine	aneugen	+	+	21%	7%	14x	2.4x	70.8	17.7-283
Bleomycin	clastogen (direct)	+	-	30%	5%	20x	1.6x	0.86	0.86-13.8
2-Nitrofluorene	clastogen (indirect)	-	-	3%	4%	1.6x	1.4x	NA	14.8-237
Mitomycin C	clastogen (direct)	+	-	21%	1%	11x	1.1x	0.02	0.00-0.24
Amlodaron	non genotoxic	-	-	3%	3%	1.3x	0.9x	NA	1.56-25.0
Diclofenac	non genotoxic	-	-	2%	4%	1.12x	1.1x	NA	6.25-100
Erythromycin	non genotoxic	-	-	2%	4%	1.1x	1.3x	NA	6.25-100
Simvastatin	non genotoxic	-	-	2%	4%	0.8x	1.1x	NA	1.56-25.0

^a Low Observable Adverse Effect Level

NA: not applicable

Clastogen (indirect): requires metabolic activation

Advantages of the Automated *In-vitro* MN Assay

Small amount of compound required: the standard *in-vitro* MN assay performed in slides requires between 10-50 mg of compound. The automated assay is performed in 96-well plates and it requires ~ 1-3 mg of compound to test at a top concentration of 200 μM.

Rapid turnaround time: manual scoring of cells is a time-consuming process which is often reflected in long turnaround times. Automated scoring is more rapid which allows for short turnaround times.

Objective Scoring: manual scoring has the potential to be biased by the subjectivity of the operator whereas automated scoring is consistently objective.

Consistent Scoring: inconsistent scoring is expected when different operators score cells manually, this problem is bypassed with automated scoring.

References:

Kirsch-Volders, M., et al., The *in vitro* micronucleus test: a multi-endpoint assay to detect simultaneously mitotic delay, apoptosis, chromosome breakage, chromosome loss and non-disjunction. *Mutat Res.* 1997; 392(1-2): p. 19-30.
 Phelps, J.B., M.L. Garriott, and W.P. Hoffman, A protocol for the *in vitro* micronucleus test. II. Contributions to the validation of a protocol suitable for regulatory submissions from an examination of 10 chemicals with different mechanisms of action and different levels of activity. *Mutat Res.* 2002; 521(1-2): p. 103-12.