

# GENETIC TOXICITY

Approval and registration of drugs requires a comprehensive assessment of their genotoxic potential. Genetic toxicology testing is an integral component of regulatory toxicology in most industrialized countries. Since no single test is capable of detecting all relevant genotoxic end-points, a battery of *in vitro* and *in vivo* tests for genotoxicity is recommended by regulatory agencies. The recommended standard test battery includes *in vitro* tests for gene mutation in bacteria (Ames test) and in mammalian cells (mouse lymphoma assay) as well as an *in vitro* test for chromosomal damage in mammalian cells (chromosomal aberration assay). Eventually, an *in vivo* test for chromosomal damage (*in vivo* micronucleus or *in vivo* chromosomal aberration assay) is required by regulatory agencies as part of an IND application.

In the recent years, 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 (miniaturized version of Ames) and the *in vitro* micronucleus assay (for the assessment of the *in vitro* chromosomal aberration assay) are routinely used for screening drug discovery compounds.

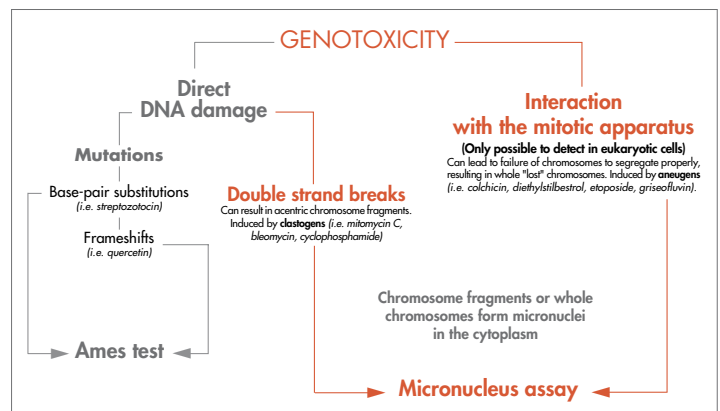
▣ MINI-AMES TA98 AND TA100 (± S9)

▣ *IN VITRO* MICRONUCLEUS (± S9)

## EARLY GENETIC TOXICITY ASSESSMENT

For early genetic toxicity assessment, Cerep offers two assays which require a small amount of test compound and are suitable for screening:

- ▴ The mini-Ames assesses the mutagenic potential of compounds.
- ▴ The *in vitro* micronucleus assay in CHOK1 cells complements the Ames test in the evaluation of genotoxic effects like chromosomal damage.



APPLICATION NOTE / ADME-TOX



# AMES TEST

## GENE MUTATION

The identification of compounds capable of inducing mutations is crucial in safety assessment, since mutagenic compounds can potentially damage the germ line and also induce cancer. Gene mutations can easily be measured in bacteria, where they cause a change in the growth requirements. The Ames test, which is conducted using *Salmonella typhimurium*, is a widely used bacterial assay for the identification of compounds that can produce gene mutations, and it shows a high predictive value with rodent carcinogenicity tests.

The Ames test typically uses 5 strains of *Salmonella* with preexisting mutations that render the bacteria unable to synthesize the essential amino-acid histidine, and are unable to grow in histidine-free medium. If a compound induces mutations on these particular genes, it can restore gene function and allow the cells to synthesize histidine and therefore grow in its absence ("reversion assay"). The *Salmonella* strains used have different mutations in various genes in the histidine operon, and are designed to be responsive to mutagenic compounds that act through different mechanisms.

## THE MINI AMES ASSAY AT CEREP

Cerep offers a miniaturized screening version of the Ames test that requires a very small amount of compound compared with the regulatory test (typically 5-10 mg). The mini-Ames is performed in 384-well plates using 2 *Salmonella* strains, TA98 and TA100. TA98 detects frameshifts and TA100 detects base substitutions leading to missense mutations. Whereas the original Ames test is carried out by plating bacteria onto selective agar plates, the mini-Ames is carried out in liquid culture using 384-well plates. The bacterial plates are incubated with the test compounds for 96 hours, after which bacterial growth is measured spectrophotometrically using a pH indicator that changes color in response to the acidification of the media due to bacterial growth.

<i>Salmonella</i> strain	DNA target	His mutation	Reversion event
TA98	CGCGCGCG	HisD3052	Frameshifts
TA100	GGG	HisG46	Base-pair substitutions

Gene mutations detected in TA98 and TA100 *Salmonella* strains

Metabolic activation is achieved using rat liver S9 fraction. To prevent false negatives due to bacteriocidal or bacteriostatic effects, a bacterial cytotoxicity assay is conducted in parallel with the mini-Ames test.

Compounds are typically tested in both bacterial strains with and without S9, at 4 concentrations (default concentrations are 5, 10, 50 and 100  $\mu\text{M}$ ; higher customized concentrations are also available upon request) with  $n = 48$  wells. A cytotoxicity test is conducted in parallel at 8 concentrations (with 100  $\mu\text{M}$  as the highest concentration) and  $n = 3$  wells. Four reference compounds (quercetin, streptozotocin, aminoanthracene and mitomycin C) are included in all experiments.

	Conc. ( $\mu\text{M}$ )	TA98	TA98+S9	TA100	TA100+S9
quercetin	10	++/+++	++/+++	-/+	-/+
streptozotocin	2.5	-/+	-/+	++/+++	++/+++
2-aminoanthracene	10	-/+	+++	-/+	+++
mitomycin C	0.15	-	-	-	-

Positive significance: if  $p < 0.05$  +  
if  $p < 0.01$  ++  
if  $p < 0.001$  +++

Reference compound data: Quercetin is the positive control for frameshift mutations, and streptozotocin for base-pair insertions/deletions. Aminoanthracene requires metabolic activation, and it ensures the quality of the S9 system. Mitomycin C prevents bacterial growth in both strains.

## ADVANTAGES OF THE MINI AMES ASSAY

▲ **SMALL AMOUNT OF COMPOUND REQUIRED**  
The regulatory Ames test requires ~200 mg of compound. The mini-Ames assay is performed in 384-well plates and it requires 5-10 mg of compound to test at a top concentration of 100  $\mu\text{M}$ .

▲ **RAPID TURNAROUND TIME**  
The results are delivered within two weeks upon receipt of the compounds at the testing site of Cerep. Data are made available on line as soon as they are validated.



# IN VITRO MICRONUCLEUS ASSAY

## MICRONUCLEUS FORMATION

Micronucleus (MN) formation is a hallmark of genetic toxicity, and the micronucleus assay is an important component of genetic toxicology screening. Micronuclei are chromatin-containing bodies that represent fragments or even whole chromosomes that were not incorporated into a daughter cell nucleus at mitosis. The purpose of the assay is to detect those agents that induce chromosome damage leading to the induction of micronuclei in interphase cells.

Micronuclei induction can result from clastogens (agents that induce chromosomal breaks mainly through interaction with the DNA) or aneugens (agents that induce

chromosomal loss mainly through interference with the spindle apparatus). The major mechanisms responsible for micronucleus induction are double DNA strand breaks leading to micronuclei with acentric fragments (chromosomal fragments lacking a centromere). The complexity of the mitotic process means that alterations can also result in the failure of the chromosomes to segregate properly. Agents that interfere with the mitotic spindle apparatus (i.e. by affecting tubulin polymerization or spindle microtubule stability) can also induce micronuclei. These micronuclei are whole chromosomes that were unable to migrate with the rest of the chromosomes during anaphase.

## PREDICTIVE VALUE OF THE *IN VITRO* MICRONUCLEUS ASSAY

The *in vitro* micronucleus assay is routinely used as a pre-screening test for the quick assessment of the *in vitro* chromosomal aberration assay, which is part of the recommended regulatory test battery for genotoxicity. Multiple published studies show a high level of concordance (80-90%) between the *in vitro*

micronucleus assay and *in vitro* chromosomal aberration assay. In addition, the *in vitro* micronucleus assay has the advantage that it is simpler, rapid, and it has more statistical power since it scores more cells; it can also detect aneuploid-inducing agents, which are very difficult to detect with the *in vitro* chromosomal aberration assay.

## THE AUTOMATED *IN VITRO* MICRONUCLEUS ASSAY

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.

## ADVANTAGES OF THE AUTOMATED *IN VITRO* MICRONUCLEUS 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 500  $\mu$ M (assuming a MW of around 500), or higher concentration at client's request.
- ▲ **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 with the results delivered within two weeks upon receipt of the compounds at the testing site of Cerep. Data are available on line as soon as they are validated.
- ▲ **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.

## THE *IN VITRO* MICRONUCLEUS ASSAY AT CEREP

The *in vitro* MN assay is conducted in CHO-K1 cells, which are widely used for this assay due to their stable and well-characterized karyotype. The cells are seeded in 96-well plates and treated with the test compounds for 24 h (without S9) and for 4 h (with S9). Cytochalasin B is added after 24 h and the cells are incubated for an additional 24 h, after which the cells are fixed

and scored for micronuclei. The assay is typically run using 10 concentrations, and data for 5 concentrations are reported. Typically, 2,000 binucleated cells are scored per concentration, or 1,000 cells per well with  $n = 2$  wells. For compounds showing very high toxicity, fewer than 2,000 cells might be scored for the toxic concentrations.

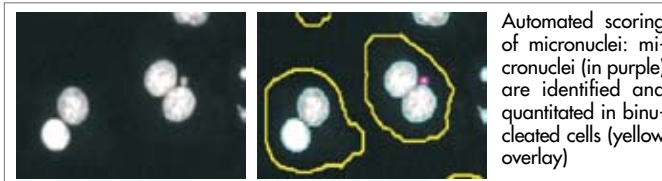


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## DETECTING MN IN THE AUTOMATED *IN VITRO* MICRONUCLEUS ASSAY

After the experimental protocol is completed, the plates are scanned with an automated fluorescent microscope (Cellomics Array Scan 7.5). The cellular nuclei and micronuclei are stained with Hoechst and the cellular cytoplasmic area is defined with a cell tracker fluorescent dye. Cellomics proprietary image-analysis software is used to identify and count micronuclei in binucleated CHO-K1 cells. A valid micronucleus should be located in the cytoplasmic cellular area,

detached and of similar intensity to the main nuclei, and with a diameter less than 33% of the main nuclei.



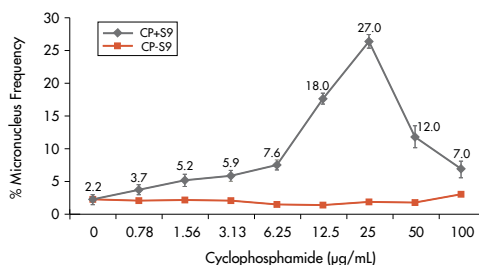
Automated scoring of micronuclei: micronuclei (in purple) are identified and quantitated in binucleated cells (yellow overlay)

## THE USE OF S9 (METABOLIC ACTIVATION)

The *in vitro* metabolic activation system consists of rat liver P450 enzymes and a NADPH regeneration system. The enzymes are contained in a preparation of liver S9 fraction from rats treated with 500 mg/kg i.p. of Aroclor 1254. The S9 fraction is obtained from a reputable supplier and maintained frozen at -70°C until use. Aliquots are freshly thawed and maintained on ice before use.

**Micronuclei frequency with cyclophosphamide treatment:** cyclophosphamide, an indirect clastogen, induces an increase of micronuclei frequency only in the presence of metabolic activation (+ S9 fraction).

Micronucleus frequency with cyclophosphamide treatment



## QUALITY CONTROLS

Each plate of customer samples includes 2 reference compounds as internal controls: mitomycin C for samples without metabolic activation, and cyclophosphamide for samples with metabolic activation. Only experiments in which reference compounds give a % of micronucleated

cells that fall within specified ranges will pass quality control. Background levels of micronucleated cells should be lower than 3% for cells without S9 and lower than 5% for cells treated with S9.

Chemical	Genotoxicity classification	Micronucleus formation		Max % cells with MN		Fold increase in MN		LOAEL <sup>1</sup> (µM)	Dose-range tested (µM)
		- S9	+ S9	- S9	+ S9	- S9	+ S9		
<b>2-Aminofluorene</b>	clastogen (indirect) <sup>2</sup>	-	-	2.3	2.4	1.5	0.9	NA <sup>3</sup>	35.0 - 552.0
<b>Colchicine</b>	aneugen	+	+	9.0	15.0	6.0	6.0	0.31	0.1 - 1.2
<b>Cyclophosphamide</b>	clastogen (indirect)	-	+	2.2	27.0	1.0	12.0	11.31	1.45 - 181.0
<b>Diethylstilbestrol</b>	aneugen	+	-	11.0	2.3	7.0	0.9	23.29	11.6 - 186.3
<b>Etoposide</b>	aneugen	+	+	22.0	13.0	14.0	5.0	0.09	0.0 - 0.3
<b>Griseofluvine</b>	aneugen	+	-	21.0	7.4	14.0	2.4	70.75	17.7 - 283.0
<b>Bleomycin</b>	clastogen (direct)	+	-	30.0	5.0	20.0	1.6	0.86	0.9 - 13.8
<b>2-Nitrofluorene</b>	clastogen (indirect)	-	-	2.5	4.2	1.6	1.4	NA	14.8 - 237.0
<b>Mitomycin C</b>	clastogen (direct)	+	-	21.0	1.3	11.0	1.1	0.02	0.0 - 0.2
<b>Amiodarone</b>	non genotoxic	-	-	2.6	2.9	1.3	0.9	NA	1.6 - 25.0
<b>Diclofenac</b>	non genotoxic	-	-	2.3	3.7	1.1	1.1	NA	6.2 - 100.0
<b>Erythromycin</b>	non genotoxic	-	-	2.3	4.3	1.1	1.3	NA	6.2 - 100.0
<b>Simvastatin</b>	non genotoxic	-	-	1.7	3.6	0.8	1.1	NA	1.6 - 25.0

<sup>1</sup> Low Observable Adverse Effect Level <sup>2</sup> Clastogen (indirect): requires metabolic activation <sup>3</sup> NA: not applicable

Micronuclei induction with a variety of chemicals: chemicals were tested at multiple concentrations, with concentration ranges determined based on the published literature

### REFERENCE:

Evaluation of an automated *in vitro* micronucleus assay in CHO-K1 cells

Diaz D., Scott A., Carmichael P., Shi W. Costales C. (2007) Mutation research, 630: 1-13

NOTE: To help in the interpretation of the results from the genotoxicity assays, Cerep also offers an aqueous solubility test (Catalog ref. 900-11a), where compound solubility is assayed up to 200 µM in an aqueous buffer (PBS pH 7.4 with 2% DMSO).



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