

GENETIC TOXICITY

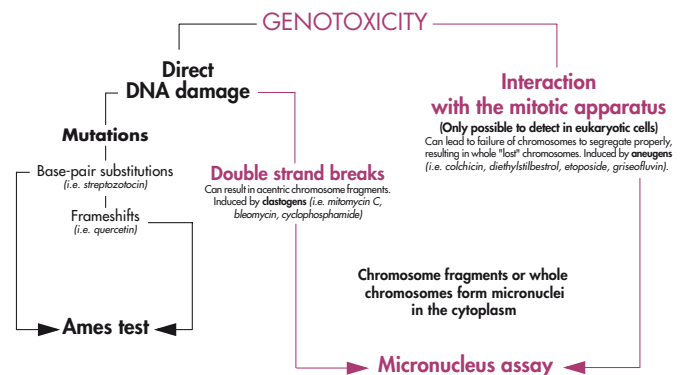
Approval and registration of drugs requires a comprehensive assessment of their genotoxic potential. Genetic toxicity testing is an integral component of regulatory toxicity in most countries. Since no single test is capable of detecting all relevant genotoxic end-points, a battery of *in vitro* and *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) or an *in vitro* test for chromosomal damage in mammalian cells (in vitro micronucleus test or *in vitro* metaphase 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, assessment of genetic toxicity testing has evolved towards earlier stages of drug discovery in order to identify genotoxic liabilities as soon as possible. Assays such as the Ames fluctuation test and the *in vitro* micronucleus assay (for the assessment of the *in vitro* chromosomal aberration) are routinely used for screening drug discovery compounds.

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



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 five strains of *Salmonella* with preexisting mutations that render the bacteria unable to synthesize the essential amino-acid histidine, and, as a result, cannot grow in histidine-free medium. If a compound induces mutations on these particular genes, it can restore gene function, allowing the cells to regain the capacity 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.

■ CEREP'S AMES FLUCTUATION ASSAY

Cerep offers a miniaturized screening version of the Ames test that requires a very small amount of compound (typically 4 mg) compared with the regulatory test. The Ames fluctuation assay is performed in 384-well plates using three *Salmonella* strains, TA98, TA100 and TA1535. TA98 detects frameshifts. TA100 detects base substitutions leading to missense mutations. TA1535 detects base substitutions and is used to identify certain mutagenic compounds which are not detected by TA98 and TA100. While the original Ames test is carried out by plating bacteria onto selective agar plates, the Ames fluctuation assay 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 |
| TA1535 | GGG | hisG46 | Base-pair substitutions |

◀ Gene mutations detected in TA98, TA100 and TA1535 *Salmonella* strains

Metabolic activation is achieved by 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 Ames fluctuation assay.

▶ GENETIC TOXICITY

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

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 fraction. Mitomycin C prevents bacterial growth in all three strains.

| Compound | Conc.(μM) | TA98 | TA98 + S9 | TA100 | TA100 + S9 | TA1535 | TA1535 + S9 |
|-----------------|------------------------|--------|-----------|-------|------------|--------|-------------|
| quercetin | 30 | ++/+++ | ++/+++ | -/+ | -/+ | - | - |
| streptozotocin | 2.5 | - | - | +++ | ++/+++ | +++ | +++ |
| aminoanthracene | 10 | - | +++ | - | +++ | - | +++ |
| mitomycin C | 0.15 | - | - | - | - | - | - |

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

■ ADVANTAGES OF THE AMES FLUCTUATION ASSAY

▶ SMALL AMOUNT OF COMPOUND REQUIRED

The regulatory Ames test requires ~200 mg of compound. The Ames fluctuation assay is performed in 384-well plates and it requires 4 mg of compound to test at a top concentration of 100 μM .

▶ 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.

■ FAQs

• What if the test compound is potentially toxic to the bacteria?

If there is a strong probability that the compound is toxic to the bacteria, the bacterial cytotoxicity assay can be run before the Ames fluctuation assay to define the appropriate test concentration for the Ames fluctuation assay. In this case, an additional five business days will be added to the turnaround time.

• What do the various flags in the bacterial cytotoxicity and Ames fluctuation assay mean?

The CYTO flag in the bacterial cytotoxicity assay indicates that there was <60% bacterial growth as compared to the negative control. The NSOL flag indicates that the test compound was not completely soluble in the working solution. This flag is applicable to both bacterial cytotoxicity and Ames fluctuation assays.

• What if the test compound is toxic to the bacteria in the bacterial cytotoxicity assay down to 10 μM ?

In this circumstance, it is recommended that the test compound is tested at lower concentrations in the Ames fluctuation assay. For example, the revised test concentrations for the compound would be 10, 5, 1, and 0.5 μM .

• Does a positive in the Ames fluctuation assay mean that the test compound will be a positive in the *in vitro* micronucleus assay?

Even though both assays assess genotoxic potential of a test compound, a positive result in one assay will not necessarily equate to a positive result in other assay largely due to the significant difference in the biological system used in each assay.

• Is Cerep's Ames test equivalent to the mini-Ames?

Actually not, it is equivalent to the AMES IITM mutagenicity assay. It is a liquid microplate modification of the Ames test which offers a higher throughput (performed in 384 well format) and consumes a lower amount of compound than the mini Ames test. It shows a good correlation with the traditional assay.

• What are the differences between strain TA100 and TA1535?

Strain TA100 is constructed by introduction of plasmid pKM101 in strain TA1535. Plasmid pKM101 enhances chemical and UV-induced mutagenesis via an increase in the error-prone recombinational DNA repair pathway. The plasmid also confers ampicillin resistance. However, it has been shown that TA1535 can identify certain chemical classes of mutagens which are not detected by TA100 (Prival, 1998, *Mutat. Res.* 412, 251-260).

IN VITRO MICRONUCLEUS ASSAY

■ MICRONUCLEUS FORMATION

Micronucleus formation is a hallmark of genetic toxicity, and the micronucleus assay is an important component of genetic toxicity 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 part of the recommended regulatory test battery for genotoxicity. It is an alternative to the *in vitro* chromosomal aberration assay. 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* micronucleus 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* micronucleus assay uses trained operators to visually read slides under a microscope, and the automated assay uses proprietary image-analysis software designed by Thermo Fisher Scientific (formerly Cellomics, Pittsburgh, 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* micronucleus assay performed in slides requires between 10-50 mg of compound. The automated assay is performed in 96-well plates and requires 1 mg of compound to test at a top concentration of 500 μ M (assuming a MW of around 500). Customized concentrations can be accommodated.

▶ 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 allowing for shorter turnaround times with the results delivered within two weeks upon receipt of the compounds at the Cerep testing site. 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.

■ CEREP'S *IN VITRO* MICRONUCLEUS ASSAY

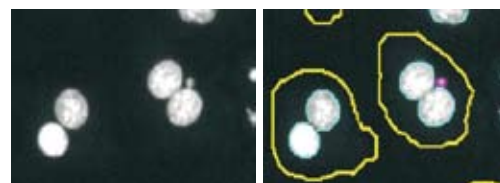
The *in vitro* micronucleus assay is conducted in CHO-K1 cells, which are widely used for this assay due to their stable and well-characterized karyotype. The cells are seeded in 96-well plates and treated with the test compounds for 24 h (without S9) and for 4 h (with S9). Cytochalasin B, which is a cytokinesis blocker, 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 in duplicate, and data for the highest 5 concentrations that are not cytotoxic 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. Duplicate wells that exhibit > 80% ($n=2$) cytotoxicity are noted as CYTO and are not scored.

■ DETECTING MICRONUCLEI IN THE AUTOMATED *IN VITRO* MICRONUCLEUS ASSAY

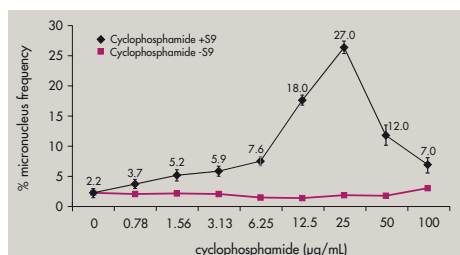
After the experimental protocol is completed, the plates are scanned with an automated fluorescent microscope (Thermo Fisher Scientific, Cellomics Array Scan 4.5). The cellular nuclei and micronuclei are stained with Hoechst and the cellular cytoplasmic area is defined with a cell tracker fluorescent dye. The micronuclei are identified and counted in bi-nucleated 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 -80°C until use. Single 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).

■ QUALITY CONTROLS

Each assay includes two reference compounds as internal controls: Mitomycin C for samples without metabolic activation ($-S9$), and cyclophosphamide for samples with metabolic activation ($+S9$). Only assays 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.

▶ GENETIC TOXICITY

Micronuclei induction with a variety of chemicals tested at multiple concentrations. Concentration ranges were determined using published literature. Cyclophosphamide and Mitomycin C are the positive controls for the +S9 and -S9 treatments, respectively ▼

| Chemical | Genotoxicity classification | Micronucleus formation | | Max % cells with MN | | Fold increase in MN | | LOAEL ¹ (µM) | Dose-range tested (µM) |
|--------------------|-----------------------------------|------------------------|------|---------------------|------|---------------------|------|-------------------------|------------------------|
| | | - S9 | + S9 | - S9 | + S9 | - S9 | + S9 | | |
| 2-Aminofluorene | clastogen (indirect) ² | - | - | 2.3 | 2.4 | 1.5 | 0.9 | NA3 | 35.0 - 552.0 |
| colchicine | aneugen | + | + | 9.0 | 15.0 | 6.0 | 6.0 | 0.31 | 0.1 - 1.2 |
| cyclophosphamide | clastogen (indirect) | - | + | 2.2 | 27.0 | 1.0 | 12.0 | 11.31 | 1.45 - 181.0 |
| diethylstilbestrol | aneugen | + | - | 11.0 | 2.3 | 7.0 | 0.9 | 23.29 | 11.6 - 186.3 |
| etoposide | aneugen | + | + | 22.0 | 13.0 | 14.0 | 5.0 | 0.09 | 0.0 - 0.3 |
| griseofluvine | aneugen | + | - | 21.0 | 7.4 | 14.0 | 2.4 | 70.75 | 17.7 - 283.0 |
| bleomycin | clastogen (direct) | + | - | 30.0 | 5.0 | 20.0 | 1.6 | 0.86 | 0.9 - 13.8 |
| 2-nitrofluorene | clastogen (indirect) | - | - | 2.5 | 4.2 | 1.6 | 1.4 | NA | 14.8 - 237.0 |
| mitomycin C | clastogen (direct) | + | - | 21.0 | 1.3 | 11.0 | 1.1 | 0.02 | 0.0 - 0.2 |
| amiodarone | non genotoxic | - | - | 2.6 | 2.9 | 1.3 | 0.9 | NA | 1.6 - 25.0 |
| diclofenac | non genotoxic | - | - | 2.3 | 3.7 | 1.1 | 1.1 | NA | 6.2 - 100.0 |
| erythromycin | non genotoxic | - | - | 2.3 | 4.3 | 1.1 | 1.3 | NA | 6.2 - 100.0 |
| simvastatin | non genotoxic | - | - | 1.7 | 3.6 | 0.8 | 1.1 | NA | 1.6 - 25.0 |

¹ Low Observable Adverse Effect Level

² Clastogen (indirect); requires metabolic activation

NA: Not Applicable

■ DATA INTERPRETATION FOR THE MICRONUCLEUS ASSAY

Test compounds are scored as either a negative (-), weak positive (-/+), or a (+) positive for clastogenic/aneugenic activity in the *in vitro* micronucleus assay. Scoring of the test compound is calculated by using the % micronucleated cells from the background. If % micronucleated cells for a test compound is < two fold of the background then the test compound is considered negative (-). Values between two-fold and three-fold are considered weak positives (-/+), while values higher than three-fold over the background are considered positive (+).

For example, if the mean % micronucleated cells for the background is 0.5% and the test compound is between 1.0% and 1.5% micronucleated cells, the compound will be considered a weak positive (-/+). If the test compounds is > 1.5%, then the compound will be considered a positive (+) in the *in vitro* micronucleus assay.

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

■ FAQs

• What do the various flags in the *in vitro* micronucleus assay mean?

There are four flags used in the *in vitro* micronucleus assay: CYTO, PREC, NSOL, and INTER. CYTO (cytotoxic) indicates that there were less than 400 scorable cells (>80% cytotoxicity) between the duplicate wells for a particular concentration. PREC indicates that precipitate was observable under the microscope. NSOL indicates that precipitate was visible with the naked eye. Both of these flags suggest that the data should be interpreted with caution. INTER indicates that test compound interferes with the high content image analysis software.

• What does it mean if the test compound is positive in the *in vitro* micronucleus assay?

False positives are inherent to all *in vitro* assays. The *in vitro* micronucleus assay is no exception. If a test compound is positive in the *in vitro* micronucleus assay, the results may be verified with additional *in vitro* genotoxicity assays or more robust *in vivo* assays, such as micronuclei formation or chromosomal aberration in rodent hematopoietic cells.



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