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OECD GUIDELINE FOR THE TESTING OF CHEMICALS

In Vitro Mammalian Cell Gene Mutation Tests using the *Hprt* and *xprt* genes

INTRODUCTION

1. The OECD Guidelines for the Testing of Chemicals are periodically reviewed in the light of scientific progress, changing regulatory needs and animal welfare. The original Test Guideline 476 (TG476) was adopted in 1984. In 1997 a revised version was adopted, based on scientific progress made to that date. This current revised version of TG476 reflects nearly thirty years of experience with this test and also results from the development of a separate new guideline dedicated to *in vitro* mammalian cell gene mutation tests using the thymidine kinase gene. TG476 is part of a series of Test Guidelines on genetic toxicology. A document that provides succinct information on genetic toxicology testing and an overview of the recent changes that were made to these Test Guidelines has been developed (1).

2. The purpose of the *in vitro* mammalian cell gene mutation test is to detect gene mutations induced by chemicals. The cell lines used in these tests measure forward mutations in reporter genes, specifically the endogeneous hypoxanthine-guanine phosphoribosyl transferase gene (*Hprt* in rodent cells, *HPRT* in human cells; collectively referred to as the *Hprt* gene and HPRT test in this Guideline), and the xanthine-guanine phosphoribosyl transferase transgene (gpt) (referred to as the XPRT test). The HPRT and XPRT mutation tests detect different spectra of genetic events. In addition to the mutational events detected by the HPRT test (e.g. base pair substitutions, frameshifts, small deletions and insertions) the autosomal location of the gpt *trans*gene may allow the detection of mutations resulting from large deletions and possibly mitotic recombination not detected by the HPRT test because the *Hprt* gene is located on the X-chromosome (2) (3) (4) (5) (6) (7). The XPRT is currently less widely used than the HPRT test for regulatory purposes.

3. Definitions used are provided in Annex 1.

INITIAL CONSIDERATIONS AND LIMITATIONS

4. Tests conducted *in vitro* generally require the use of an exogenous source of metabolic activation. The exogenous metabolic activation system does not entirely mimic *in vivo* conditions.

5. Care should be taken to avoid conditions that would lead to artifactual positive results, (*i.e.* possible interaction with the test system), not caused by direct interaction between the test chemicals and the genetic material of the cell; such conditions include changes in pH or osmolality (8) (9) (10), interaction with the medium components (11) (12), or excessive levels of cytotoxicity (13). Cytotoxicity

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exceeding the recommended top cytotoxicity levels as defined in paragraph 19 is considered excessive for the HPRT test.

6. Before use of the Test Guideline on a mixture for generating data for an intended regulatory purpose, it should be considered whether, and if so why, it may provide adequate results for that purpose. Such considerations are not needed when there is a regulatory requirement for testing of the mixture.

PRINCIPLE OF THE TEST

7. Mutant cells deficient in Hprt enzyme activity in the HPRT test or xprt enzyme activity in the XPRT test are resistant to the cytostatic effects of the purine analogue 6-thioguanine (TG). The Hprt (in the HPRT test) or gpt (in XPRT test) proficient cells are sensitive to TG, which causes the inhibition of cellular metabolism and halts further cell division. Thus, mutant cells are able to proliferate in the presence of TG, whereas normal cells, which contain the Hprt (in the HPRT test) or gpt (in XPRT test) enzyme, are not.

8. Cells in suspension or monolayer cultures are exposed to the test chemical, both with and without an exogenous source of metabolic activation (see paragraph 14), for a suitable period of time (3-6 hours), and then sub-cultured to determine cytotoxicity and to allow phenotypic expression prior to mutant selection (14) (15) (16) (17). Cytotoxicity is determined by relative survival (RS), i.e. cloning efficiency measured immediately after treatment and adjusted for any cell loss during treatment as compared to the negative control (paragraph 18 and Annex 2). The treated cultures are maintained in growth medium for a sufficient period of time, characteristic of each cell type, to allow near-optimal phenotypic expression of induced mutations (typically a minimum of 7-9 days). Following phenotypic expression, mutant frequency is determined by seeding known numbers of cells in medium containing the selective agent to detect mutant colonies, and in medium without selective agent to determine the cloning efficiency (viability). After a suitable incubation time, colonies are counted. Mutant frequency is calculated based on the number of mutant colonies corrected by the cloning efficiency at the time of mutant selection.

DESCRIPTION OF THE METHOD

Preparations

Cells

9. The cell types used for the HPRT and XPRT tests should have a demonstrated sensitivity to chemical mutagens, a high cloning efficiency, a stable karyotype, and a stable spontaneous mutant frequency. The most commonly used cells for the HPRT test include the CHO, CHL and V79 lines of Chinese hamster cells, L5178Y mouse lymphoma cells, and TK6 human lymphoblastoid cells (18) (19). CHO-derived AS52 cells containing the *gpt* transgene (and having the *Hprt* gene deleted) are used for the XPRT test (20) (21); the HPRT test cannot be performed in AS52 cells because the hprt gene has been deleted. The use of other cell lines should be justified and validated.

10. Cell lines should be checked routinely for the stability of the modal chromosome number and the absence of *Mycoplasma* contamination (22) (23), and cells should not be used if contaminated or if the modal chromosome number has changed. The normal cell cycle time used in the testing laboratory should be established and should be consistent with the published cell characteristics. The spontaneous mutant

frequency in the master cell stock should also be checked, and the stock should not be used if the mutant frequency is not acceptable.

11. Prior to use in this test, the cultures may need to be cleansed of pre-existing mutant cells, e.g.by culturing in HAT medium for HPRT test and MPA for XPRT test (5) (24) (See Annex 1). The cleansed cells can be cryopreserved and then thawed to use as working stocks. The newly thawed working stock can be used for the test after normal doubling times are attained. When conducting the XPRT test, routine culture of AS52 cells should use conditions that assure the maintenance of the *gpt* transgene (20).

Media and culture conditions

12. Appropriate culture medium and incubation conditions (culture vessels, humidified atmosphere of 5% CO_2, and incubation temperature of 37°C) should be used for maintaining cultures. Cell cultures should always be maintained under conditions that ensure that they are growing in log phase. It is particularly important that media and culture conditions be chosen to ensure optimal growth of cells during the expression period and optimal cloning efficiency for both mutant and non-mutant cells.

Preparation of cultures

13. Cell lines are propagated from stock cultures, seeded in culture medium at a density such that the cells in suspensions or in monolayers will continue to grow exponentially through the treatment and expression periods (e.g. confluence should be avoided for cells growing in monolayers).

Metabolic activation

14. Exogenous metabolising systems should be used when employing cells which have inadequate endogenous metabolic capacity. The most commonly used system, that is recommended by default, unless otherwise justified, is a co-factor-supplemented post-mitochondrial fraction (S9) prepared from the livers of rodents (generally rats) treated with enzyme-inducing agents such as Aroclor 1254 (25) (26) (27) (28) or a combination of phenobarbital and β-naphthoflavone (29) (30) (31) (32). The latter combination does not conflict with the Stockholm Convention on Persistent Organic Pollutants (33) and has been shown to be as effective as Aroclor 1254 for inducing mixed-function oxidases (29) (31). The S9 fraction typically is used at concentrations ranging from 1 to 2% (v/v) but may be increased to 10% (v/v) in the final test medium. The choice of the type and concentration of exogenous metabolic activation system or metabolic inducer employed may be influenced by the class of substances being tested (34) (35) (36).

Test chemical Preparation

15. Solid test chemicals should be prepared in appropriate solvents and diluted, if appropriate, prior to treatment of the cells (see paragraph 16). Liquid test chemicals may be added directly to the test system and/or diluted prior to treatment of the test system. Gaseous or volatile test chemicals should be tested by appropriate modifications to the standard protocols, such as treatment in sealed culture vessels (37) (38). Preparations of the test chemical should be made just prior to treatment unless stability data demonstrate the acceptability of storage.

Test conditions

Solvents

16. The solvent should be chosen to optimize the solubility of the test chemicals without adversely impacting the conduct of the test e.g. changing cell growth, affecting the integrity of the test chemical, reacting with culture vessels, impairing the metabolic activation system. It is recommended that, wherever possible, the use of an aqueous solvent (or culture medium) should be considered first. Well established solvents are for example, water and dimethyl sulfoxide. Generally, organic solvents should not exceed 1% (v/v) and aqueous solvents (saline or water) should not exceed 10% (v/v) in the final treatment medium. If the solvents used are not well-established (e.g. ethanol or acetone), their use should be supported by data indicating their compatibility with the test chemicals and the test system, and their lack of genetic toxicity at the concentration used. In the absence of that supporting data, it is important to add untreated controls (see Annex 1) to demonstrate that no deleterious or mutagenic effects are induced by the chosen solvent.

Measuring cytotoxicity and choosing exposure concentrations

17. When determining the highest test chemical concentration, concentrations that have the capability of producing artifactual positive responses, such as those producing excessive cytotoxicity (see paragraph 20), precipitation in the culture medium (see paragraph 21), or marked changes in pH or osmolality (see paragraph 5) should be avoided. If the test chemical causes a marked change in the pH of the medium at the time of addition, the pH might be adjusted by buffering the final treatment medium so as to avoid artifactual positive results and to maintain appropriate culture conditions.

18. Concentration selection is based on cytotoxicity and other considerations (see paragraphs 20-22). While the evaluation of cytotoxicity in an initial test may be useful to better define the concentrations to be used in the main experiment, an initial test is not required. Even if an initial cytotoxicity evaluation is performed, the measurement of cytotoxicity for each culture is still required in the main experiment. Cytotoxicity should be evaluated using RS, i.e. cloning efficiency (CE) of cells plated immediately after treatment, adjusted by any loss of cells during treatment, based on cell count, as compared with adjusted cloning efficiency in negative controls (assigned a survival of 100%) (see Annex 2 for the formula).

19. At least four test concentrations (not including the solvent and positive controls) that meet the acceptability criteria (appropriate cytotoxicity, number of cells, etc.) should be evaluated. While the use of duplicate cultures is advisable, either replicate or single treated cultures may be used at each concentration tested. The results obtained in the independent replicate cultures at a given concentration should be reported separately but can be pooled for the data analysis (17). For test chemicals demonstrating little or no cytotoxicity, concentration intervals of approximately 2 to 3 fold will usually be appropriate. Where cytotoxicity occurs, the test concentrations selected should cover a range from that producing cytotoxicity to concentrations at which there is moderate and little or no cytotoxicity. Many test chemicals exhibit steep concentration response curves and in order to cover the whole range of cytotoxicity or to study the concentration response relationship in detail, it may be necessary to use more closely spaced concentrations and more than four concentrations, in particular in situations where a repeat experiment is required (see paragraph 43). The use of more than 4 concentrations may be particularly important when using single cultures.

20. If the maximum concentration is based on cytotoxicity, the highest concentration should aim to achieve between 20 and 10% RS. Care should be taken when interpreting positive results only found at 10% RS or below (paragraph 43).

21. For poorly soluble test chemicals that are not cytotoxic at concentrations below the lowest insoluble concentration, the highest concentration analysed should produce turbidity or a precipitate visible by eye or with the aid of an inverted microscope at the end of the treatment with the test chemical. Even if cytotoxicity occurs above the lowest insoluble concentration, it is advisable to test at only one concentration producing turbidity or with a visible precipitate because artifactual effects may result from the precipitate. At the concentration producing a precipitate, care should be taken to assure that the precipitate does not interfere with the conduct of the test. The determination of solubility in the culture medium prior to the experiment may be useful.

22. If no precipitate or limiting cytotoxicity is observed, the highest test concentration should correspond to 10 mM, 2 mg/mL or 2 μL/mL, whichever is the lowest (39) (40). When the test chemical is not of defined composition, e.g. substance of unknown or variable composition, complex reaction products or biological materials (i.e. Chemical Substances of Unknown or Variable Composition (UVCBs)) (41), environmental extracts, etc., the top concentration may need to be higher (e.g. 5 mg/mL), in the absence of sufficient cytotoxicity, to increase the concentration of each of the components. It should be noted however that these requirements may differ for human pharmaceuticals (42).

Controls

23. Concurrent negative controls (see paragraph 16), consisting of solvent alone in the treatment medium and handled in the same way as the treatment cultures, should be included for every experimental condition.

24. Concurrent positive controls are needed to demonstrate the ability of the laboratory to identify mutagens under the conditions of the test protocol used and the effectiveness of the exogenous metabolic activation system, when applicable. Examples of positive controls are given in Table 1 below. Alternative positive control substances can be used, if justified. Because *in vitro* mammalian cell tests for genetic toxicity are sufficiently standardized, tests using treatments with and without exogenous metabolic activation may be conducted using only a positive control requiring metabolic activation. In this case, this single positive control response will demonstrate both the activity of the metabolic activation system and the responsiveness of the test system. Each positive control should be used at one or more concentrations expected to give reproducible and detectable increases over background in order to demonstrate the sensitivity of the test system, and the response should not be compromised by cytotoxicity exceeding the limits specified in the TG (see paragraph 20).

Table 1. Reference substances recommended for assessing laboratory proficiency and for selection of positive controls.

Metabolic Activation condition	Locus	Substance and CAS No.
Absence of exogenous metabolic activation	*Hprt*	Ethylmethanesulfonate [CAS no. 62-50-0] Ethylnitrosourea [CAS no. 759-73-9] 4-Nitroquinoline 1-oxide [CAS no. 56-57-5]
	xprt	Streptonigrin [CAS no. 3930-19-6] Mitomycin C [CAS no. 50-07-7]
Presence of exogenous metabolic activation	*Hprt*	3-Methylcholanthrene [CAS no. 56-49-5] 7,12-Dimethylbenzanthracene [CAS no. 57-97-6] Benzo[a]pyrene [CAS no. 50-32-8]
	xprt	Benzo[a]pyrene [CAS no. 50-32-8]

PROCEDURE

Treatment with test chemical

25. Proliferating cells are treated with the test chemical in the presence and absence of a metabolic activation system. Exposure should be for a suitable period of time (usually 3 to 6 hours is adequate).

26. The minimum number of cells used for each test (control and treated) culture at each stage in the test should be based on the spontaneous mutant frequency. A general guide is to treat and passage sufficient cells as to maintain 10 spontaneous mutants in every culture in all phases of the test (17). The spontaneous mutant frequency is generally between 5 and 20 $x10^{-6}$. For a spontaneous mutant frequency of 5 $x10^{-6}$ and to maintain a sufficient number of spontaneous mutants (10 or more) even for the cultures treated at concentrations that cause 90% cytotoxicity during treatment (10% RS), it would be necessary to treat at least 20 x 10^6 cells. In addition a sufficient number of cells (but never less than 2 million) must be cultured during the expression period and plated for mutant selection (17).

Phenotypic expression time and measuring mutant frequency

27. After the treatment period, cells are cultured to allow expression of the mutant phenotype. A minimum of 7 to 9 days generally is sufficient to allow near optimal phenotypic expression of newly induced *Hprt* and *xprt* mutants (43) (44). During this period, cells are regularly sub-cultured to maintain them in exponential growth. After phenotypic expression, cells are re-plated in medium with and without selective agent (6-thioguanine) for the determination of the number of mutants and cloning efficiency at the time of selection, respectively. This plating can be accomplished using dishes for monolayer cultures or microwell plates for cells in suspension. For mutant selection, cells should be plated at a density to assure optimum mutant recovery (i.e. avoid metabolic cooperation) (17). Plates are incubated for an appropriate length of time for optimum colony growth (e.g. 7-12 days) and colonies counted. Mutant

frequency is calculated based on the number of mutant colonies corrected by the cloning efficiency at the time of mutant selection (see Annex 2 for formulas).

Proficiency of the laboratory

28. In order to establish sufficient experience with the test prior to using it for routine testing, the laboratory should have performed a series of experiments with reference positive substances acting via different mechanisms (at least one active with and one active without metabolic activation selected from the substances listed in Table 1) and various negative controls (using various solvents/vehicles). These positive and negative control responses should be consistent with the literature. This is not applicable to laboratories that have experience, *i.e.* that have an historical data base available as defined in paragraphs 30 to 33.

29. A selection of positive control substances (see Table 1 in paragraph 25) should be investigated in the absence and in the presence of metabolic activation, in order to demonstrate proficiency to detect mutagenic substances, to determine the effectiveness of the metabolic activation system and to demonstrate the appropriateness of the cell growth conditions during treatment, phenotypic expression and mutant selection and of the scoring procedures. A range of concentrations of the selected substances should be chosen so as to give reproducible and concentration-related increases above the background in order to demonstrate the sensitivity and dynamic range of the test system.

Historical control data

30. The laboratory should establish:

- A historical positive control range and distribution,
- A historical negative (untreated, solvent) control range and distribution.

31. When first acquiring data for an historical negative control distribution, concurrent negative controls should be consistent with published control data (22). As more experimental data are added to the control distribution, concurrent negative controls should ideally be within the 95% control limits of that distribution (17) (45) (46).

32. The laboratory's historical negative control database should initially be built with a minimum of 10 experiments but would preferably consist of at least 20 experiments conducted under comparable experimental conditions. Laboratories should use quality control methods, such as control charts (e.g. C-charts or X-bar charts (47)), to identify how variable their positive and negative control data are, and to show that the methodology is 'under control' in their laboratory (46). Further recommendations on how to build and use the historical data (i.e. criteria for inclusion and exclusion of data in historical data and the acceptability criteria for a given experiment) can be found in the literature (45).

33. Negative control data should consist of mutant frequencies from single or preferably replicate cultures as described in paragraph 23. Concurrent negative controls should ideally be within the 95% control limits of the distribution of the laboratory's historical negative control database (17) (45) (46). Where concurrent negative control data fall outside the 95% control limit they may be acceptable for inclusion in the historical control distribution as long as these data are not extreme outliers and there is evidence that the test system is 'under control' (see above) and there is evidence of no technical or human failure.

34. Any changes to the experimental protocol should be considered in terms of their consistency with the laboratory's existing historical control databases. Any major inconsistencies should result in the establishment of a new historical control database.

DATA AND REPORTING

Presentation of the results

35. The presentation of results should include all of the data needed to calculate cytotoxicity (expressed as RS). The data, for both treated and control cultures, should include the number of cells at the end of treatment, the number of cells plated immediately following treatment, and the colony counts (or number of wells without colonies for the microwell method). RS for each culture should be expressed as a percentage relative to the concurrent solvent control (refer to Annex 1 for definitions).

36. The presentation of results should also include all of the data needed to calculate the mutant frequency. Data for both treated and control cultures, should include: (1) the number of cells plated with and without selective agent (at the time the cells are plated for mutant selection), and (2) the number of colonies counted (or the number of wells without colonies for the microwell method) from the plates with and without selective agent. Mutant frequency is calculated based on the number of mutant colonies (in the plates with selective agent) corrected by the cloning efficiency (from the plates without selective agent). The mutant frequency should be expressed as the number of mutant cells per million viable cells (refer to Annex 1 for definitions).

37. Individual culture data should be provided. Additionally, all data should be summarised in tabular form.

Acceptability Criteria

38. Acceptance of a test is based on the following criteria:

- The concurrent negative control is considered acceptable for addition to the laboratory historical negative control database as described in paragraph 33.
- Concurrent positive controls (see paragraph 24) should induce responses that are compatible with those generated in the historical positive control data base and produce a statistically significant increase compared with the concurrent negative control.
- Two experimental conditions (i.e. with and without metabolic activation) were tested unless one resulted in positive results (see paragraph 25).
- Adequate number of cells and concentrations are analysable (paragraphs 25, 26 and 19).
- The criteria for the selection of top concentration are consistent with those described in paragraphs 20, 21 and 22.

Evaluation and interpretation of results

39. Providing that all acceptability criteria are fulfilled, a test chemical is considered to be clearly positive if, in any of the experimental conditions examined:

a) at least one of the test concentrations exhibits a statistically significant increase compared with the concurrent negative control,

b) the increase is concentration-related when evaluated with an appropriate trend test,

c) any of the results are outside the distribution of the historical negative control data (e.g. Poisson-based 95% control limit; see paragraph 33).

When all of these criteria are met, the test chemical is then considered able to induce gene mutations in cultured mammalian cells in this test system. Recommendations for the most appropriate statistical methods can be found in the literature (46) (48).

40. Providing that all acceptability criteria are fulfilled, a test chemical is considered clearly negative if, in all experimental conditions examined:

a) none of the test concentrations exhibits a statistically significant increase compared with the concurrent negative control,

b) there is no concentration-related increase when evaluated with an appropriate trend test,

c) all results are inside the distribution of the historical negative control data (e.g. Poisson-based 95% control limit; see paragraph 33).

The test chemical is then considered unable to induce gene mutations in cultured mammalian cells in this test system.

41. There is no requirement for verification of a clearly positive or negative response.

42. In cases when the response is neither clearly negative nor clearly positive as described above, or in order to assist in establishing the biological relevance of a result, the data should be evaluated by expert judgement and/or further investigations. Performing a repeat experiment possibly using modified experimental conditions (e.g. concentration spacing, other metabolic activation conditions [i.e. S9 concentration or S9 origin]) could be useful.

43. In rare cases, even after further investigations, the data set will preclude making a conclusion of positive or negative results. Therefore the test chemical response should be concluded to be equivocal (interpreted as equally likely to be positive or negative).

Test report

44. The test report should include the following information:

Test chemical:

- source, lot number, limit date for use, if available;
- stability of the test chemical itself, if known;
- solubility and stability of the test chemical in solvent, if known;
- measurement of pH, osmolality and precipitate in the culture medium to which the test chemical was added, as appropriate.

Mono-constituent substance:

- physical appearance, water solubility, and additional relevant physicochemical properties;

- chemical identification, such as IUPAC or CAS name, CAS number, SMILES or InChI code, structural formula, purity, chemical identity of impurities as appropriate and practically feasible, etc.

Multi-constituent substance, UVBCs and mixtures:

- characterised as far as possible by chemical identity (see above), quantitative occurrence and relevant physicochemical properties of the constituents.

Solvent:

- justification for choice of solvent;
- percentage of solvent in the final culture medium.

Cells:

For Laboratory master cultures:

- type, source of cell lines;
- number of passages, if available, and history in the laboratory;
- karyotype features and/or modal number of chromosomes;
- methods for maintenance of cell cultures;
- absence of mycoplasma;
- cell doubling times.

Test conditions:

- rationale for selection of concentrations and number of cultures including, e.g. cytotoxicity data and solubility limitations;
- composition of media, CO_2 concentration, humidity level;
- concentration of test chemical expressed as final concentration in the culture medium (e.g. µg or mg/mL or mM of culture medium);
- concentration (and/or volume) of solvent and test chemical added in the culture medium;
- incubation temperature;
- incubation time;
- duration of treatment;
- cell density during treatment;
- type and composition of metabolic activation system (source of S9, method of preparation of the S9 mix, the concentration or volume of S9 mix and S9 in the final culture medium, quality controls of S9);
- positive and negative control substances, final concentrations for each condition of treatment;
- length of expression period (including number of cells seeded, and subcultures and feeding schedules, if appropriate);
- identity of the selective agent and its concentration;
- criteria for acceptability of tests;
- methods used to enumerate numbers of viable and mutant cells;
- methods used for the measurements of cytotoxicity;
- any supplementary information relevant to cytotoxicity and method used;
- duration of incubation times after plating;
- criteria for considering studies as positive, negative or equivocal;
- methods used to determine pH, osmolality and precipitation.

Results:

- number of cells treated and number of cells sub-cultured for each culture;
- cytotoxicity measurements and other observations if any;
- signs of precipitation and time of the determination;
- number of cells plated in selective and non-selective medium;
- number of colonies in non-selective medium and number of resistant colonies in selective medium, and related mutant frequencies;
- concentration-response relationship, where possible;
- concurrent negative (solvent) and positive control data (concentrations and solvents);
- historical negative (solvent) and positive control data, with ranges, means and standard deviations and confidence interval (e.g. 95%) as well as the number of data;
- statistical analyses (for individual cultures and pooled replicates if appropriate), and p-values if any.

Discussion of the results.

Conclusion.

LITERATURE

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ANNEX 1

DEFINITIONS

Base pair substitution mutagens: substances that cause substitution of base pairs in the DNA.

Cloning efficiency: The percentage of cells plated at a low density that are able to grow into a colony that can be counted.

Concentrations: refer to final concentrations of the test chemical in culture medium

Cytotoxicity: For the assays covered in this guideline, cytotoxicity is identified as a reduction in relative survival of the treated cells as compared to the negative control (see specific paragraph).

Forward mutation: a gene mutation from the parental type to the mutant form which gives rise to an alteration or a loss of the enzymatic activity or the function of the encoded protein.

Frameshift mutagens: substances which cause the addition or deletion of single or multiple base pairs in the DNA molecule.

Genotoxic: a general term encompassing all types of DNA or chromosomal damage, including DNA breaks, adducts, rearrangements, mutations, chromosome aberrations, and aneuploidy. Not all types of genotoxic effects result in mutations or stable chromosomal damage

HAT medium: medium containing Hypoxanthine, Aminopterin and Thymidine, used for cleansing of Hprt mutants.

Mitotic recombination: during mitosis, recombination between homologous chromatids possibly resulting in the induction of DNA double strand breaks or in a loss of heterozygosity.

MPA medium: medium containing Xanthine, Adenine, Thymidine, Aminopterin and Mycophenolic acid, used for cleansing of Xprt mutants.

Mutagenic: produces a heritable change of DNA base-pair sequences(s) in genes or of the structure of chromosomes (chromosome aberrations).

Mutant frequency (MF): the number of mutant colonies observed divided by the number of cells plated in selective medium, corrected for cloning efficiency (or viability) at the time of selection.

Phenotypic expression time: The time after treatment during which the genetic alteration is fixed within the genome and any preexisting gene products are depleted to the point that the phenotypic trait is altered.

Relative survival (RS): RS is used as the measure of treatment-related cytotoxicity. RS is cloning efficiency (CE) of cells plated immediately after treatment adjusted by any loss of cells during treatment as compared with cloning efficiency in negative controls (assigned a survival of 100%).

S9 liver fractions: supernatant of liver homogenate after 9000g centrifugation, i.e. raw liver extract

S9 mix: mix of the liver S9 fraction and cofactors necessary for metabolic enzyme activity.

Solvent control: General term to define the control cultures receiving the solvent alone used to dissolve the test chemical.

Untreated control: cultures that receive no treatment (i.e. no test chemical nor solvent) but are processed concurrently and in the same way as the cultures receiving the test chemical

ANNEX 2

FORMULAS FOR ASSESSMENT OF CYTOTOXICITY AND MUTANT FREQUENCY

Cytotoxicity is evaluated by relative survival, i. e., cloning efficiency (CE) of cells plated immediately after treatment adjusted by any loss of cells during treatment as compared with adjusted cloning efficiency in negative controls (assigned a survival of 100%) (see RS formula below).

Adjusted CE for a culture treated by a test chemical is calculated as:

$$\text{Adjusted CE} = \text{CE} \times \frac{\text{Number of cells at the end of treatment}}{\text{Number of cells at the beginning of treatment}}$$

RS for a culture treated by a test chemical is calculated as:

$$\text{RS} = \frac{\text{Adjusted CE in treated culture}}{\text{Adjusted CE in the solvent control}} \times 100$$

Mutant frequency is the cloning efficiency of mutant colonies in selective medium divided by the cloning efficiency in non-selective medium measured for the same culture at the time of selection.

$$\text{Mutant frequency} = \frac{\text{Cloning efficiency of mutant colonies in selective medium}}{\text{Cloning efficiency in non-selective medium.}}$$

When plates are used for cloning efficiency:

CE = Number of colonies / Number of cells plated.

When micro-well plates are used for cloning efficiency:

The number of colonies per well on micro-wells plates follows a Poisson distribution.

Cloning Efficiency = -LnP(0) / Number of cells plated per well

Where -Ln P(0) is the probable number of empty wells out of the seeded wells and is described by the following formula

LnP(0) = -Ln (number of empty wells / number of plated wells)