Designation: E 1262 – 88 (Reapproved 1996)

# Standard Guide for Performance of the Chinese Hamster Ovary Cell/ Hypoxanthine Guanine Phosphoribosyl Transferase Gene Mutation Assay[1]

This standard is issued under the fixed designation E 1262; the number immediately following the designation indicates the year of original adoption or, in the case of revision, the year of last revision. A number in parentheses indicates the year of last reapproval. A superscript epsilon (ε) indicates an editorial change since the last revision or reapproval.

## 1. Scope

1.1 This guide highlights some of the more relevant biological concepts as they are currently understood, and summarizes the critical technical aspects for acceptable bioassay performances as they currently are perceived and practiced. The Chinese hamster ovary cell/hypoxanthine guanine phosphoribosyl transferase (CHO/HGPRT) assay **(1)**[2] has been widely applied to the toxicological evaluation of industrial and environmental chemicals.

1.2 This guide concentrates on the practical aspects of cell culture, mutagenesis procedures, data analysis, quality control, and testing strategy. The suggested approach represents a consensus of the panel members for the performance of the assay. It is to be understood, however, that these are merely general guidelines and are not to be followed without the use of sound scientific judgement. Users of the assay should evaluate their approach based on the properties of the substances to be tested and the questions to be answered.

1.3 Deviation from the guidelines based on sound scientific judgement should by no means invalidate the results obtained.

1.4 *This standard does not purport to address all of the safety concerns, if any, associated with its use. It is the responsibility of the user of this standard to establish appropriate safety and health practices and determine the applicability of regulatory limitations prior to use.*

## 2. Significance and Use

2.1 The CHO/HGPRT assay detects forward mutations of the X-linked hypoxanthine-guanine phosphoribosyl transferase (hgprt) locus (coding for the enzyme, HGPRT) in Chinese hamster ovary (CHO) cells. Cells originally derived from Chinese hamster ovary tissue are exposed to a test article and, following an appropriate cell culture regimen, descendants of the original treated population are monitored for the loss of functional HGPRT, presumably due to mutations. Resistance to a purine analogue, 6-thioguanine (6TG) (or less desirably, 8-azaguanine (8AG)), is employed as the genetic marker. HGPRT catalyzes the conversion of the nontoxic 6TG to its toxic ribophosphorylated derivative. Loss of the enzyme or its activity therefore leads to cells resistant to 6TG.

2.2 Because HGPRT is an enzyme of the purine nucleotide salvage pathway, loss of the enzyme is not a lethal event. Different types of mutational events (base substitutions, frameshifts, deletions, some chromosomal type lesions, etc.) should theoretically be detectable at the hgprt locus. The CHO/HGPRT assay has been used to study a wide range of mutagens, including radiations **(2-4)**, and a wide variety of chemicals **(1)**, and complex chemical mixtures **(5)**.

## 3. Characteristics of CHO Cells

3.1 Different CHO cell lines/subclones are appropriate for the CHO/HGPRT assay. The CHO-K1-BH4 cell line developed and extensively characterized by **(6)** is probably the most widely employed. The CHO(WT) cell line and its derivative, CHO-AT3-2, are used to monitor mutations at other gene loci in addition to hgprt **(7, 8)**. While there are differences among the cell lines employed, a number of general characteristics are critical for the performance of the assay:

3.1.1 The cloning efficiency (CE) of the stock cultures should not be less than 70 %. The CE of untreated or solvent control experimental cultures should not be less than 50 %.

3.1.2 Cultures in logarithmic phase of growth should have a population doubling time of 12 to 16 h.

3.1.3 The modal chromosome number should be 20 or 21, as is characteristic of the particular cell line/subclone used.

3.1.4 Cultures should be free from microbial and mycoplasma contamination.

3.2 The cell properties that are critical for the assay should be routinely monitored as part of the quality control regimen. Routine quality control procedures should include testing of

[1] This guide is under the jurisdiction of ASTM Committee F04 on Medical and Surgical Materials and Devices and is the direct responsibility of Subcommittee F04.16 on Biocompatibility Test Methods.

Current edition approved July 29, 1988. Published September 1988.

[2] The boldface numbers in parentheses refer to the list of references at the end of this guide.

serum and media for each new purchase, as well as mycoplasma and karyotype checks at least once yearly, preferably once every three months.

## 4. Mutagenesis Procedures

4.1 The mutagenesis protocol can be divided into three phases: mutagen treatment, expression, and selection.

4.2 *Mutagen Treatment*:

4.2.1 *Cell Plating*—Cells should be in exponential phase when plated for treatment. Several media (for example, Ham's F12, alpha-MEM) that are known to be optimal for cell growth can be used. Cells should be seeded at an appropriate cell density to allow exponential growth as well as quantitation of induced responses. A common practice is to plate $0.5 \times 10^6$ cells in a 25–$cm^2$ flask, or $1.5 \times 10^6$ cells in a 75–$cm^2$ flask, on the day before treatment.

4.2.2 *Chemical Handling*—The solubility of the test article in an appropriate medium should be determined before treatment. Commonly used solvents are, in the order of preference, medium, water, dimethylsulfoxide, ethanol, and acetone. Generally, the nonaqueous solvent concentration should not exceed 1 % and should be constant for all samples. As part of the solubility test, an aliquot of the test chemical should be added to the treatment medium to note any pH changes, the presence of any chemical precipitation, and any apparent reaction of the chemical or solvent with the culture vessel. The solvent of choice should not have any undesirable reactions with the test article, culture vessel, or cells.

4.2.3 *Addition of Test Article to Cells*—Stock solutions of the test samples are prepared and aliquots are added to each flask. Dilutions of the test article should be such that the concentration of solvent remains constant for all samples. Cells are generally treated with the test article for at least 3 h. For treatment times of 3 to 5 h, serum–free medium can be used. As serum is required to maintain cell division, medium containing serum should be used for a prolonged treatment period (for example, 16 h or longer). Serum requirement for treatment periods between 5 and 16 h should be determined on a case-by-case basis.

4.2.4 *Exogenous Activation Systems*—Aroclor 1254-induced rat liver homogenate (S9) is the most commonly used exogenous metabolic activating system for the assay. When S9 is used, cofactors for the mixed function monooxygenases should be present. Calcium chloride ($CaCl_2$), which enhances the mutagenicity of nitrosamines and polycyclic hydrocarbons **(9, 10)**, appears to be another useful addition. However, the need for $CaCl_2$ has yet to be documented for a wide variety of chemicals. A commonly used cofactor mixture consists of sodium phosphate (50 m*M*, pH 7.0 to 8.0), NADP (4 m*M*), glucose-6-phosphate (5 m*M*), potassium chloride (30 m*M*), magnesium chloride (10 m*M*), and $CaCl_2$ (10 m*M*). S9 is added directly to the cofactor mixture. One volume of the S9/cofactor mixture is added to 4 volumes of the treatment medium. Other exogenous systems (for example, hepatocytes, S9 from other animal species or produced using different enzyme induction conditions, and other cofactor mixtures) can also be used depending on the intent of the experiment.

4.2.5 *Estimation of Cytotoxicity*—Plating CHO cells immediately after treatment for cytotoxicity determination is generally expected to yield the most accurate results. Otherwise, cytotoxicity can be estimated on the day after treatment. Aliquots of the cells are plated to allow for colony development. Cytotoxicity is usually expressed as relative CE which is the ratio of the CE of the treated cells to that of the solvent control. Viability determination should take into account any loss of cells during the treatment period, cell trypsinization procedures, and the overnight incubation period.

4.2.6 *Positive and Solvent Controls*—An appropriate negative control is treatment of cells with the solvent used for the test article. Positive controls, both direct-acting and indirect-acting, should also be included to demonstrate the adequacy of the experimental conditions to detect known mutagens. An untreated control may also be included to evaluate the effects of the solvent on mutagenicity. Commonly used positive controls are ethyl methane sulfonate (EMS) and *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine (MNNG) as direct-acting mutagens, and benzo(a)pyrene (BaP) and dimethylnitrosamine (DMN) as promutagens that require metabolic activation.

4.3 *Expression of Induced Mutations*:

4.3.1 After mutation at the hgprt locus, the mutant phenotype requires a period of time before it is completely expressed (expression requires the loss of pre-existing enzyme activity). Phenotypic expression is presumably achieved by dilution of the pre-existing HGPRT enzyme and mRNA through cell division and macromolecular turnover. At the normal population doubling times of 12 to 16 h for CHO cells, an expression period of 7 to 9 days is generally adequate **(11, 12)**.

4.3.2 The most widely employed method for phenotypic expression allows exponential growth of the cells for a defined time period after mutagen treatment. CHO cells can be subcultured with 0.05 % trypsin with or without EDTA. Aliquots of $1 \times 10^6$ cells are subcultured at 2 or 3 day intervals in 100–mm diameter tissue culture dishes or 75 $cm^2$ *t*-flasks. Either complete medium or hypoxanthine-free medium can be employed, with either dialyzed or nondialyzed serum. It is important to ensure that the medium employed will allow a population doubling time of 12 to 16 h.

4.3.3 Besides the normal growth of cells as monolayer cultures, alternative methods of subculturing involving suspension **(8)**, unattached **(13)**, and division arrested **(14)** cultures have also been successful. The use of a particular subculture regimen in the expression period should be substantiated by data demonstrating the achievement of optimal expression.

4.4 *Mutant Selection*:

4.4.1 Conditions for the selection of mutants must be defined to ensure that only mutant cells are able to form colonies and that there is no significant reduction in the ability of mutant cells to form colonies. In general, cells are plated in tissue culture dishes for attached colony growth **(11)**, or in agar for suspended colony growth **(16)**. An advantage of the former is that after the colonies are fixed and stained, the plates can be counted at a later date. An advantage of the latter is that metabolic cooperation between wild type and mutant cells is reduced, allowing selection of a higher cell number per plate. For attached colonies, the cells are in general cultured for a period of 6 to 8 days and the number of colonies counted after fixing (for example, with 10 % formalin or 70 % methanol),

and staining (for example, with 10 % Giemsa or crystal violet). Soft agar colonies are usually counted *in situ* after a culturing period of 10 to 14 days.

4.4.2 Reliable selection has been established in hypoxanthine-free medium containing dialyzed serum and 10 μ*M* 6TG. Fetal bovine serum, newborn bovine serum, or calf serum can be used, providing that the serum has been adequately tested and shown to support the desirable characteristics of CHO cells as described here. Dialyzed serum is usually necessary to eliminate the competition between 6TG and purine bases in the serum. It has been found that a selection cell density of $2 \times 10^5$ or fewer cells per 100 mm dish for attached colony growth **(14, 15)** and $10^6$ or fewer cells per 100 mm dish (in 30 mL of agar) for agar colony growth **(16)** allows essentially 100 % recovery of mutant cells.

## 5. Data Presentation

5.1 Results from the assay should include the following experimental data:

5.1.1 Concentrations and solvents used for the test article and positive controls.

5.1.2 Absolute and relative cloning efficiencies (CE) in the concurrent cytotoxicity assay.

5.1.2.1 *Absolute CE*—Absolute CE equals the number of colonies formed divided by the number of cells plated.

5.1.2.2 *Relative CE*—Relative CE equals CE (treatment) divided by CE (solvent control).

5.1.3 Actual number of mutant colonies observed for each treatment condition.

5.1.4 Absolute CE at selection for each treatment condition.

5.1.5 Mutant frequency (MF) values, expressed as mutants per $10^6$ cells.

5.1.5.1 *Mutant Frequency (MF) Values*—MF values equal the number of mutant colonies divided by the number of clonable cells.

5.1.5.2 *Number of Clonable Cells*—The number of clonable cells equals the cells plated multiplied by the absolute CE at selection.

## 6. Criteria for Data Acceptability

6.1 Generally, for the data of a given assay to be acceptable, the following criteria should be met:

6.1.1 The absolute CE of the negative controls should not be less than 50 %. Absolute CE values lower than 50 % would indicate suboptimal culturing conditions for the cells.

6.1.2 The mean mutant frequency of the solvent controls in each experiment should fall within the range from 0 to 20 mutants per $10^6$ clonable cells. A higher mutant frequency may preclude detection of weak mutagens. Under such conditions data acceptability should be evaluated on a case–by–case basis.

6.1.3 The positive control must induce a statistically significant response at a magnitude appropriate for the mutagen under the chosen experimental conditions.

6.1.4 The highest test article concentration should, if possible, result in a significant cytotoxic response (for example, 10 % to 30 % survival, where survival is the percent of the treated population that is viable after treatment). This is particularly important if the response is negative. For noncytotoxic test articles, the highest concentration has generally been 1 to 10 mg/mL, or to the limit of solubility.

## 7. Data Analysis

7.1 Due to the possibility of stochastic fluctuation, only samples with no fewer than 100 000 viable cells after treatment should be used for data analysis. Judgement on mutagenicity should be made based on the following information:

7.1.1 Dose response relationship.

7.1.2 Significance of response (in comparison to the negative control).

7.1.3 Reproducibility of the results.

7.2 Exact statistical analysis is difficult because the distribution of the number of mutant colonies depends on the complex processes of cell growth and death after mutagen treatment. While other appropriate methods can be used, the following two approximate methods are used commonly:

7.2.1 *Weighted Regression Analysis*—A weighted regression analysis where the weights are proportional to the observed number of mutant colonies divided by the square of the observed mutant frequency **(17)**. This weighting scheme was derived by assuming that the variance of the observed mutant frequency is a constant multiple of that which would occur if the number of mutant colonies on each selection plate per treatment conforms to a Poisson distribution. A test compound is considered to exhibit a mutagenic response if the slope of the mutant induction as a function of test concentrations is greater than zero at the 0.01 level according to the *t*-test **(18)**.

7.2.2 *Power Transformation Procedure*—A power transformation procedure with which the observed mutant frequency is transformed using the following equation:

$$Y = (X + \alpha)^{\beta} \quad (1)$$

where:

$Y$ = transformed mutant frequency,
$X$ = observed mutant frequency, and
$\alpha, \beta$ = constants.

7.2.2.1 Data transformed by this method appears to satisfy the assumptions of homogeneous variance and normal distribution **(18)**. Comparison to negative control values and dose response relationships are examined with Student's *t*-test and an analysis of variance (ANOVA) using the transformed values. Computations can be done with computer programs readily available.

## 8. Testing Strategy

8.1 In general, the mutagenicity test should be designed to consider the following:

8.1.1 The test substance should be tested at levels allowing significant chemical-cell interaction, which is generally indicated by cytotoxicity at the highest useful dose levels. Relatively insoluble chemicals should be tested to at least the limit of solubility. Nontoxic but highly soluble chemicals should be tested to an arbitrary maximum concentration based on the anticipated human exposure level and a conservative safety factor. As a general rule of thumb, 1 to 10 mg/mL should be sufficient as the maximum concentration.

8.1.2 Different amounts of Aroclor 1254-induced liver S9 may be used, since it has been shown that some mutagens may be highly sensitive to the level of S9 used **(9, 10)**.

8.1.3 The observation should be reproducible as indicated by two or more independent experiments.

8.1.4 In each experiment, intra-experimental variations should be determined using replicate treatment cultures.

8.1.5 An example of an adequate combination of experiments **(19)** is as follows:

8.1.5.1 *Experiment 1*—Range-finding for cytotoxicity. Log or half-log concentrations of the test articles are evaluated in the absence and presence of various levels of S9. Cytotoxicity information obtained is used for dose selection in the subsequent mutagenesis experiments. A repeat of the experiment using a narrower concentration range may be necessary for test articles with steep cytotoxic responses.

8.1.5.2 *Experiment 2*—Initial mutagenicity determination with limited doses and at multiple S9 concentrations. This experiment should yield information for an initial estimation of mutagenicity as well as any effects of S9 concentration on mutagenicity. Concentrations of the test article are selected based on the results of Experiment 1.

8.1.5.3 *Experiment 3*—Confirmatory mutagenicity determination. This experiment would incorporate a single S9 level, optimized if possible using data from Experiment 2. A larger number of concentrations than in Experiment 2 should be used for a more accurate estimation of dose-response relationship, if any.

8.2 General guidelines for the performance of this assay for chemical testing have also been published, and can be used as a basis for experimental design, for example, **(1, 21, 22, 23, 24)**.

## 9. Other Considerations

9.1 This guide should not be viewed as encompassing the only available, appropriate, or useful protocols and procedures. There is no substitute for sound scientific judgement and "hands on" experience. This guide, therefore, should not be construed as an instrument for inhibiting present or future research and development towards further refinement of the assay.

9.2 Being an extensively characterized assay, the CHO/HGPRT assay should be useful in the toxicological evaluation of industrial and environmental substances. An advantage of employing CHO cells is that other well-characterized genotoxicity endpoints have also been developed in this cell system, for example, mutations at other gene loci **(7, 8, 25, 26, 27)**, chromosomal aberrations **(28)**, and sister-chromatid-exchanges **(29)**. It is therefore possible to use a variety of endpoints in CHO cells for testing, yielding additional information that may be used, in conjunction with data from other toxicity assays, for the prediction of the human toxicological consequences of exposure to the substances tested.

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