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

This standard is issued under the fixed designation E1262; 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 The values stated in SI units are to be regarded as standard. No other units of measurement are included in this standard.

1.5 *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, and so forth) 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

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[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 0.5×10^6 cells in a 25-cm^2 flask, or 1.5×10^6 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 1×10^6 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