

OECD GUIDELINE FOR THE TESTING OF CHEMICALS

DRAFT PROPOSAL FOR A NEW GUIDELINE XXX:

Transgenic Rodent Gene Mutation Assays

INTRODUCTION

1. OECD guidelines are available for a wide range of *in vitro* mutation assays that are able to detect chromosomal and/or gene mutations. However, while there are Test Guidelines for some *in vivo* somatic cell some endpoints (i.e. chromosomal aberrations and unscheduled DNA synthesis), aside from the little-used (Wahnschaffe *et al.*, 2005) rodent Spot Test (TG 484), a Test Guideline for detecting gene mutations *in vivo* is not available.. *Transgenic* Rodent (TGR) mutation assays fulfill the need for a practical *in vivo* test for gene mutations, and will serve as a well-validated and more widely available alternative for the Spot Test. Additional, transgenic rodent gene mutation assays use many fewer animals and conform to the 3Rs principles of animal usage (reduction, refinement and replacement).
2. The TGR assays have been reviewed extensively (OECD, 2009). The Mammalian *Transgenic* Rodent (TGR) assays use transgenic rats and mice that contain multiple copies of chromosomally integrated plasmid or phage *shuttle vectors*. The transgenes contain *reporter genes* for the detection of gene mutations and/or chromosomal rearrangements (plasmid model and Spi⁻ assay) induced *in vivo* by test substances.
3. Mutations arising in a rodent are scored by recovering the shuttle vector and analysing the phenotype of the reporter gene in a bacterial host deficient for the reporter gene. TGR gene mutation assays measure mutations induced in genetically *neutral marker genes* recovered from virtually any tissue of the rodent. These assays, therefore, circumvent many of the existing limitations associated with the study of *in vivo* gene mutation in endogenous genes (e.g., limited tissues available for analysis, negative/positive selection against mutations).
4. The weight of evidence suggests that transgenes respond to mutagens in a similar manner to *endogenous genes*, especially with regard to the detection of *base pair substitutions, frameshift mutations, and small deletions and insertions* (OECD, 2009).
5. The International Workshops on Genotoxicity Testing (IWGT) have endorsed the inclusion of TGR gene mutation assays for *in vivo* detection of gene mutations, and has recommended a protocol for their implementation (Heddle *et al.*, 2000; Thybaud *et al.*, 2003). This Test Guideline is based on these reports. Further analysis supporting the use of this protocol can be found in Heddle *et al.*, 2003.
6. Definitions of terms in italics are set out in the Annex.

INITIAL CONSIDERATIONS

7. TGR models for which sufficient data are available to support their use in this Test Guideline are: *lacZ* bacteriophage mouse (MutaTMMouse); *lacZ* plasmid mouse; *gpt* delta (*gpt* and Spi⁻) mouse and rat assays; *lacI* mouse and rat assays (Big Blue[®]), as performed under standard conditions. In addition, the

cII positive selection assay can be used for evaluating mutations in the Big Blue[®] and Muta[™]Mouse models. New TGR gene mutation assays may be accepted in the future if they meet OECD acceptance and validation criteria. Mutagenesis in the TGR models is normally assessed as mutant frequency; if required, however, molecular analysis of the mutations can provide additional information (see paragraphs 22).

8. These rodent *in vivo* gene mutation tests are especially relevant to assessing mutagenic hazard in that the assays' responses are dependent upon *in vivo* metabolism, pharmacokinetics, DNA repair processes, and translesion DNA synthesis, although these may vary among species, among tissues and among the types of DNA damage. An *in vivo* assay for gene mutations is useful for further investigation of a mutagenic effect detected by an *in vitro* system, and for following up results of tests using other *in vivo* endpoints. In addition to being causally associated with the induction of cancer, gene mutation is a relevant endpoint for the prediction of mutation-based non-cancer diseases in somatic tissues (Erikson, 2003; 2010) as well as in the germline.

9. If there is no evidence produced that the test substance, or a reactive metabolite, will reach a particular tissue under the test conditions, it is not appropriate to use negative results from this tissue.

PRINCIPLE OF THE TEST METHOD

10. In the assays described in paragraph 7 the target gene is bacterial or bacteriophage in origin, and the means of recovery from the rodent genomic DNA is by incorporation of the transgene into a λ bacteriophage or plasmid shuttle vector. The procedure involves the extraction of genomic DNA from the rodent tissue of interest, *in vitro* processing of the genomic DNA (i.e. *packaging* of λ vectors, or *ligation* and *electroporation* of plasmids) to recover the shuttle vector, and subsequent detection of mutations in bacterial hosts under suitable conditions. The included assays employ neutral transgenes that are readily recoverable from most tissues.

11. The basic TGR experiment involves treatment of the rodent with a substance over a period of time via toxicologically relevant modes of administration. Agents may be administered by any appropriate route, including implantation (e.g. medical device testing). The total period during which an animal is dosed is referred to as the *administration time*. Administration is followed by a period of time, prior to sacrifice, during which the agent is not administered and during which unrepaired DNA lesions are fixed into stable mutations. In the literature, this period has been variously referred to as the *sampling time*, *manifestation time*, *fixation time* or *expression time*; in this document, this period is referred to as the *sampling time*, in keeping with the terminology adopted by the IWGT (Heddle *et al.*, 2000; Thybaud *et al.*, 2003). After the animal is sacrificed, genomic DNA is isolated from the tissue(s) of interest and purified.

12. An essential feature of the TGR assay is that mutation detection is achieved *in vitro* following the rescue of reporter gene vectors from the genomic DNA by *in vitro* packaging of the λ shuttle vectors or excision/religation of integrated plasmids. Data for a single tissue per animal from multiple packaging/ligations are usually aggregated, and mutant frequency is generally evaluated using a total of between 10^5 and 10^7 *plaque-forming* or *colony-forming units*. When using positive selection methods, total plaque-forming units are determined with a separate set of nonselective plates.

13. Positive selection methods have been developed to facilitate the detection of mutations in both the *gpt* gene (*gpt* delta mouse and rat, *gpt*⁻ phenotype) (Nohmi *et al.*, 1996; Nohmi, Suzuki and Masumura, 2000; Toyoda-Hokaiwado *et al.*, 2010) and the *lacZ* gene (Muta[™]Mouse or *lacZ* plasmid mouse) (Gossen *et al.*, 1989; Gossen and Vijg, 1993; Boerrigter *et al.*, 1995; Vijg and Douglas, 1996); whereas, *lacI* gene

mutations in Big Blue® animals are detected through a nonselective method that identifies mutants through the generation of coloured (blue) plaques. Methodology is also in place to detect point mutations arising in the *cII* gene of the λ bacteriophage shuttle vector (Big Blue® mouse or rat, and Muta™ Mouse; Jakubczak *et al.*, 1996; Nohmi, Suzuki and Masumura, 2000) and deletion mutations in the λ *red* and *gam* genes (Spi^- selection in *gpt* delta mouse and rat; Nohmi *et al.*, 1999; Nohmi, Suzuki and Masumura, 2000; Toyoda-Hokaiwado *et al.*, 2010). *Mutant frequency* is calculated by dividing the number of plaques/plasmids containing mutations in the transgene by the total number of plaques/plasmids recovered from the same DNA sample. In most TGR mutation studies the mutant frequency is reported. A *mutation frequency* can be determined as the fraction of cells carrying independent mutations; this calculation requires correction for *clonal expansion* by sequencing the recovered mutants (OECD, 2009).

14. The mutations scored in the *lacI*, *lacZ*, *cII* and *gpt* point mutation assays consist primarily of *base pair substitution* mutations, *frameshift* mutations and small *insertions/deletions*. The relative proportion of these mutation types among spontaneous mutations is similar to that seen in the endogenous *Hprt* gene. *Large deletions* are exclusively detected with the Spi^- selection and the *lacZ* plasmid assays. Mutations of interest are *in vivo* mutations that arise in the mouse or rat. *In vitro* and *ex vivo* mutations, which may arise during phage/plasmid recovery, replication or repair, are uncommon, and in some systems can be specifically identified, or excluded by the bacterial host/positive selection system.

DESCRIPTION OF THE METHOD

Preparations

Selection of animal species

15. A variety of transgenic mouse gene mutation detection models are currently available, and these systems have been more widely used than transgenic rat models. If the rat is clearly a more appropriate model than the mouse (*e.g.* when investigating the mechanism of carcinogenesis for a tumour seen only in rats, or to correlate with a rat toxicity study) the use of transgenic rat models should be considered.

Housing and feeding conditions

16. The temperature in the experimental animal room should ideally be 22°C ($\pm 3^\circ\text{C}$). Although the relative humidity should be at least 30% and preferably not exceed 70% other than during room cleaning, the goal should be to maintain a relative humidity of 50-60%. Lighting should be artificial, with a daily sequence of 12 hours light, followed by 12 hours dark. For feeding, conventional laboratory diets may be used with an unlimited supply of drinking water. The choice of diet may be influenced by the need to ensure a suitable admixture of a test substance when administered by this route. Animals may be housed individually, or caged in small groups of the same sex if no aggressive behavior is expected.

Preparation of the animals

17. Healthy young adult animals are randomly assigned to the control and treatment groups. The animals are identified uniquely. The animals are acclimated to the laboratory conditions for at least five days. Cages should be arranged in such a way that possible effects due to cage placement are minimized. At the commencement of the study, the weight variation of animals should be minimal and not exceed $\pm 20\%$ of the mean weight of each sex

Preparation of doses

18. Solid test substances should be dissolved or suspended in appropriate solvents or vehicles and diluted, if appropriate, prior to dosing of the animals. Liquid test substances may be dosed directly or diluted prior to dosing. Fresh preparations of the test substance should be employed unless stability data demonstrate the acceptability of storage. Serial dilutions of suspensions should be avoided.

Test Conditions

Solvent/vehicle

19. The solvent/vehicle should not produce toxic effects at the dose levels used, and should not be suspected of chemical reaction with the test substance. If other than well-known solvents/vehicles are used, their inclusion should be supported with reference data indicating their compatibility. It is recommended that wherever possible, the use of an aqueous solvent/vehicle should be considered first.

Controls

20. Concurrent positive control animals are recommended. However, for laboratories that have demonstrated competency in these assays, concurrent positive control animals are not strictly necessary, although their inclusion is desirable. Competency in these assays can be validated by demonstrating published norms for expected results with concurrent positive controls (including weak mutagens) and non-mutagens, replication of published norms for transgene recovery from genomic DNA (e.g., packaging efficiency), and vehicle control and induced mutant frequency (OECD, 2009). If a concurrent positive control group is not used, positive control DNA should be included with each study to confirm the success of the method. In such cases, positive control DNA from the competency phase can be used. When concurrent positive controls are used they should be administered by the same route as the test substance. Examples of positive control substances include the following.

Chemical and CAS No.	Characteristics	Somatic Mutation Target Tissue	
		Rat	Mouse
N-Ethyl-N-nitrosourea [CAS no. 759-73-9]	Strong, direct acting mutagen	Liver, lung	Bone marrow, colon, colonic epithelium, intestine, liver, lung, spleen, ovarian granulosa cells
Ethyl carbamate (urethane) [CAS no. 51-79-6]	Weak mutagen, requires metabolism	No data	Bone marrow, forestomach, liver, lung, spleen
Benzo[a]pyrene [CAS no. 50-32-8]	Strong mutagen, requires metabolism	Liver, omenta	Bone marrow, breast, colon, forestomach, glandular stomach, heart

21. Negative controls, treated with solvent or vehicle alone, and otherwise treated in the same way as the treatment groups should be included for every sampling time. In the absence of historical or published control data showing that no deleterious or mutagenic effects are induced by the chosen solvent/vehicle, untreated controls should also be included.

Sequencing of mutants

22. For regulatory applications, DNA sequencing of mutants is not required, particularly where a clear positive or negative result is obtained. However, sequencing data may be useful when high inter-individual variation is observed. In these cases, sequencing can be used to rule out the possibility of *jackpots* or clonal events by identifying the proportion of unique mutants from a particular tissue. Sequencing up to 10 mutants per tissue should be sufficient for simply determining if clonal mutants contribute to the mutant frequency; sequencing as many as 25 mutants may be necessary to correct mutant

frequency mathematically for clonality. Sequencing of mutants also may be considered when low mutant frequencies (i.e. just exceeding the untreated control values) are found. Differences in the mutant spectrum between the mutant colonies from treated and untreated animals may lend support to a mutagenic effect (Adams and Skopek, 1987). Also, mutation spectra may be useful for developing mechanistic hypotheses. When sequencing is to be included as part of the study protocol, special care should be taken in the design of such studies, in particular with respect to the number of mutants sequenced per sample to achieve adequate power according to the statistical model used.

PROCEDURE

Number and Sex of Animals

23. The number of animals per group must be sufficient to provide statistical power sufficient to detect a doubling or less in mutant frequency. Assays should use groups of at least 5 animals; if the statistical power is insufficient, the number of animals can be increased up to 10 animals per group. Male animals should normally be used, consistent with OECD Test Guideline recommendations for other *in vivo* genotoxicity tests. However, if there are significant differences between the sexes in terms of toxicity or metabolism, then both males and females will be required. There may be cases where testing females alone would be justified; for example, when testing human female-specific drugs, or when investigating female-specific metabolism. These recommendations are applicable to the rat as well as to the mouse.

Administration Time

24. Based on observations that mutations accumulate with each treatment, a repeated-dose regimen is strongly encouraged, with daily treatments for a period of 28 days generally considered adequate both for producing a sufficient accumulation of mutations by weak mutagens and for providing an adequate exposure time adequate for detecting mutations in slowly proliferating organs. Alternative treatment regimens, such as weekly dose administration, may be appropriate for some evaluations, and these alternative dosing schedules should be scientifically justified in the protocol, particularly if negative results are obtained. Treatments should not be shorter than the time required for the complete induction of all the relevant metabolising enzymes, and shorter treatments may necessitate the use of multiple sampling times that are suitable for organs with different proliferation rates. In any case, all available information e.g. on general toxicity or metabolism and pharmacokinetics should be used when justifying a protocol, especially when deviating from the above standard recommendations. While it may increase sensitivity, treatment times longer than 8 weeks should be explained clearly and justified, since long treatment times may produce an apparent increase in mutant frequency through clonal expansion (Thybaud et al., 2003).

Dose Levels

25. Dose levels should be based on the results of a dose range finding study measuring general toxicity that was conducted by the same route of exposure, or the result of pre-existing sub-acute toxicity studies. Non-transgenic animals of a related rodent strain may be used for dose ranging. In the main test, a complete study must include a negative control group and a minimum of three dose levels, except where the limit dose has been used (see paragraph 26). The top dose should be the Maximum Tolerated Dose (MTD). The MTD is defined as the dose producing signs of toxicity such that higher dose levels, based on the same dosing regimen, would be expected to produce lethality. Substances with specific biological activities at low non-toxic doses (such as hormones and *mitogens*) may be exceptions to the dose-setting criteria and should be evaluated on a case-by-case basis. The dose levels used should cover a range from the maximum to little or no toxicity. Analysis of at least three treated dose groups is recommended in order to provide sufficient data for dose response analysis.

Limit Test

26. If dose range-finding experiments, or existing data from related rodent strains, indicate that a treatment regime of at least the limit dose (see below) produces no observable toxic effects, and if genotoxicity would not be expected based upon data from structurally related substances, then a full study using three dose levels may not be considered necessary. Since an administration time of 28 days (i.e. 28 daily treatments) is recommended (see Paragraph 24), the OECD standard limit dose for this administration time is 1000 mg/kg body weight/day (i.e. for administration times of 14 days or more). For administration times less than 14 days in duration, the limit dose is 2000 mg/kg/body weight/day. Expected human exposure may, in rare instances, indicate the need for a higher dose level than the limit dose.

Administration of Doses

27. The test substance is usually administered by gavage using a stomach tube or a suitable intubation cannula. Other routes of exposure (such as, drinking water, subcutaneous, intravenous, topical, inhalation, intratracheal, or implantation) may be acceptable where they can be justified. Intraperitoneal injection should be avoided. In general, the route of human exposure should be considered when designing an assay for an unknown agent. The maximum volume of liquid that can be administered by gavage or injection at one time depends on the size of the test animal. The volume should not exceed 2 ml/100g body weight. The use of volumes greater than these must be justified. Except for irritating or corrosive substances, which will normally reveal exacerbated effects at higher concentrations, variability in test volume should be minimised by adjusting the concentration to ensure a constant volume at all dose levels.

Sampling Time: Somatic Cells

28. The sampling time is a critical variable because it is the time needed for mutations to be fixed, and for the mutant frequency to reach a plateau or maximum. This period is tissue-specific and appears to be related to the turnover time of the cell population, with bone marrow and intestine being rapid responders and the liver being much slower. A suitable compromise for the measurement of mutant frequencies in both rapidly and slowly proliferating tissues is 28 consecutive daily treatments (as indicated in Paragraph 24) and sampling 3 days after the final treatment; although the maximum mutant frequency may not manifest itself in slowly proliferating tissues under these conditions. If slowly proliferating tissues are of particular importance, then a longer sampling time of 28 days following the 28 day administration period may be more appropriate (Thybaud *et al.*, 2003). In such cases, the longer sampling time would replace the 3 day sampling time, and would require scientific justification.

Sampling Time: Germ Cells

TGR assays are well-suited for the study of gene mutation induction in male germ cells (Douglas *et al.*, 2005; 2006; Singer *et al.*, 2006), in which the timing and kinetics of spermatogenesis have been well-defined (Singer *et al.*, 2006). However, the low numbers of ova available for analysis, even after superovulation, and the fact that there is no DNA synthesis in the oocyte, preclude the determination of mutation in female germ cells using transgenic assays (Yauk *et al.*, 2005).

The sampling times for male germ cells should be selected so that the range of exposed cell types throughout germ cell development are sampled, and the exposure of the stage targeted in the sampling has received the sufficient exposure. The time for the progression of developing germ cells from the end of the spermatogonial stem cell phase to mature sperm reaching the vas deferens is ~41 days for the mouse and ~57 days for the rat (Singer *et al.*, 2006). After a 28 day exposure, accumulated sperm collected from the vas deferens (Douglas *et al.*, 1996) after a subsequent 3 day sampling period will represent a population of cells exposed during approximately the latter half of spermatogenesis, which includes the meiotic and postmeiotic period, but not the spermatogonial or stem cell period. In order to accurately sample cells that were spermatogonial stem cells during the exposure period, a 56 day sampling time is

required in addition to the 3 day sampling period. These sampling times are sufficient for both mouse and rat germ cells.

In addition to cells sampled from the vas deferens at these times (3 and 56 days), cells extruded from seminiferous tubules after 28 days of exposure comprise a mixed population enriched for all stages of developing germ cells (Douglas *et al.*, 1995; 1996) which enables an assessment of spermatogonial stem cell mutations induced during the 28 day exposure period. This method does not provide as precise an assessment of the stages at which germ cell mutations are induced that can be obtained from sampling spermatozoa from the vas deferens (since there a range of cell stages sampled, and there will be some somatic cells contaminating the cell population). However, if cells are harvested from the tubuli at the 3 day sampling time when spermatozoa are sampled from the vas deferens, the detection of mutations induced in spermatogonial stem cells and in the early stages of spermatogenesis is enabled (Douglas *et al.*, 1995). If confirmation of the specific stage of origin of induced mutations is needed, a subsequent experiment using a 56 day sampling time in which sperm are collected from the vas deferens could be considered.

Accordingly, mutation induced in all stages of male germ cell development can be detected after a 28 day exposure by sampling spermatozoa from the vas deferens and developing germ cells from the seminiferous tubules of the same animals after a single 3 day sampling time. If a more precise estimate of mutations induced in stem cells is needed, it can be obtained by performing a subsequent experiment with a 56 day sampling time and assaying sperm collected from the vas deferens.

Observations

29. General clinical observations should be made at least once a day OECD (2000), preferably at the same time(s) each day and considering the peak period of anticipated effects after dosing. The health condition of the animals should be recorded. At least twice daily, all animals are observed for morbidity and mortality. All animals should be weighed at least once a week. Measurements of food consumption should be made at least weekly. If the test substance is administered via the drinking water, water consumption should also be measured at least weekly. Animals exhibiting non-lethal indicators of excess toxicity should be euthanatized prior to completion of the test period (OECD, 2000)

Tissue Selection

30. The rationale for tissue selection should be defined clearly. Since it is possible to study mutation induction in virtually any tissue, the selection of tissues to be sampled should be based upon the reason for conducting the study and any existing mutagenicity, carcinogenicity or toxicity data for the compound under investigation. Important factors for consideration should include the route of administration (based on likely human exposure route(s)), the predicted tissue distribution, and the possible mechanism of action. In the absence of any background information, it is recommended that at least three tissues be sampled in the experiment. At least one rapidly dividing (*e.g.* bone marrow) and one slowly dividing tissue (*e.g.* liver) should be evaluated initially for mutagenicity. If a compound is negative in these two tissues, a third tissue sampled in the study may also be evaluated. The choice of a third tissue would be based on considerations such as: 1) the route of administration or site of first contact (*e.g.* glandular stomach if administration is oral, lung if the administration is through inhalation; 2) skin if topical application has been used); and 3) pharmacokinetic parameters observed in general toxicity studies, which indicate tissue disposition, retention or accumulation, or target organs for toxicity. The choice of the third tissue should maximize the detection of compounds that are direct-acting *in vitro* mutagens, rapidly metabolized, highly reactive or poorly absorbed, or those for which the target tissue is determined by route of administration (Dean *et al.*, 1999). If studies are conducted as follow-up of carcinogenicity studies, target tissues for carcinogenicity should be considered.

Storage of Tissues and DNA

31. Tissues should be stored at or below -70°C and be used for DNA isolation within 5 years. Isolated DNA, stored refrigerated at 4°C in appropriate buffer, should be used optimally for mutation analysis within 1 year..

Methods of Measurement

32. Standard laboratory or published methods for the detection of mutants are available for the recommended transgenic models: *lacZ* lambda bacteriophage and plasmid (Vijg and Douglas, 1996); *LacI* mouse (Kohler et al., 1990); *gpt* delta mouse (Nohmi, Suzuki and Masumura, 2000); *gpt* delta rat (Toyoda-Hokaiwado et al, 2010). Modifications should be justified and properly documented. There is no biological justification to set a minimum acceptable number of plaque-forming units or colony-forming units from an individual packaging: all data can be used and aggregated to reach the optimal number of plaques or colonies. However, if a large number of packaging reactions is needed to reach the appropriate number of plaques, it could be due to poor DNA quality. In such cases, data should be considered cautiously because the data may be unreliable. The optimal total number of plaques or colonies per DNA sample is governed by the statistical probability of detecting sufficient numbers of mutants at a given spontaneous mutant frequency. In general, a minimum of 125,000 to 300,000 plaques is required if the spontaneous mutant frequency is in the order of 3×10^{-5} (Heddle et al., 2000). Tissues and the resulting samples (items) should be processed and analysed using a block design, where items from the vehicle/solvent control group, the positive control group (if used) or positive control DNA (where appropriate), and each treatment group are processed together.

DATA AND REPORTING

Treatment of Results

33. Regulatory studies should be conducted according to Good Laboratory Practices. Individual animal data should be presented in tabular form. The experimental unit is the animal. The report should include the total number of plaque-forming units or colony-forming units, the number of mutants, and the mutant frequency for each tissue from each animal. If there are multiple packaging/rescue reactions the number per DNA sample should be reported, although data for each individual reaction should be retained, but only the total pfu or cfu need be reported. Data on toxicity and clinical sign as per paragraph 30 should be reported. Any sequencing results should be presented for each mutant analyzed, and resulting mutation frequency calculations for each animal and tissue should be shown.

Statistics

33. While biological relevance of the results should be the ultimate consideration, statistical methods may be used as an aid in evaluating the test results. A number of appropriate statistical approaches have been developed for transgenic rodent gene mutation assay data (Piegorsch et al., 1995, 1997; Carr and Gorelick, 1995; Fung et al, 1998; Heddle et al., 2000). Statistical tests used should consider the animal as the experimental unit. There are several criteria for determining a positive result, such as a concentration-related, or a reproducible increase in mutant frequency. A number of statistical approaches available to assist in interpreting the results of DNA sequencing analyses (Adams and Skopek, 1987; Carr and Gorelick, 1996; Dunson and Tindall, 2000).

34. A positive result is one in which the data for one or more tissues show a statistically significant, and/or biologically relevant, dose-dependent increase in mutant frequency, or a statistically significant and/or biologically relevant increase in mutant frequency in any dose group as compared with concurrent vehicle/solvent controls.

35. A negative result is one in which a statistically significant and/or biologically relevant dose-dependent increase in mutant frequency is not found in any of the tissues studied at levels up to the, MTD, the limit dose, or tumorigenic dose, and in which the mean mutant frequencies at all doses are within two standard deviations of the mean mutant frequency of the concurrent negative (vehicle) control.

Test report

36. The test report should also include the following information:

Test substance:

- identification data and CAS no., if known;
- physical nature and purity;
- physiochemical properties relevant to the conduct of the study;
- stability of the test substance, if known.

Solvent/vehicle:

- justification for choice of vehicle;
- solubility and stability of the test substance in the solvent/vehicle, if known;
- preparation of dietary or drinking water formulations;
- analytical determinations (e.g., stability, homogeneity, nominal concentrations) target.

Test animals:

- species/strain used and justification for the choice;
- number, age and sex of animals;
- source, housing conditions, diet, etc.;
- individual weight of the animals at the start of the test, including body weight range, mean and standard deviation for each group.

Test conditions:

- positive and negative (vehicle/solvent) control data;
- data from the range-finding study;
- rationale for dose level selection;
- details of test substance preparation;
- details of the administration of the test substance;
- rationale for route of administration;
- methods for measurement of animal toxicity, including, where available, histopathological or hematological analyses and the frequency with which animal observations and body weights were taken;
- methods for verifying that the test substance reached the target tissue, or general circulation, if applicable;
- actual dose (mg/kg body weight/day) calculated from diet/drinking water test substance concentration (ppm) and consumption, if applicable;
- details of food and water quality;
- detailed description of treatment and sampling schedules and justifications for the choices;
- method of euthanasia;
- methods for isolation of rodent genomic DNA, rescuing the transgene from genomic DNA, and transferring transgenic DNA to a bacterial host;
- Source and lot numbers of all cells, kits and reagents (where applicable);
- methods for enumeration of mutants;

- methods for molecular analysis of mutants and use in correcting for clonality and/or calculating mutation frequencies, if applicable.

Results:

- animal condition prior to and throughout the test period, including signs of toxicity;
- for each tissue/animal the number of mutants, number of plaques or colonies evaluated, mutant frequency;
- for each tissue/animal group, total number of plaques or colonies evaluated, number of packaging reactions per DNA sample, total number of mutants, mean mutant frequency, standard error;
- dose-response relationship, where possible;
- for each tissue/animal, the number of independent mutants and mean mutation frequency, where molecular analysis of mutations was performed;
- statistical analyses and methods applied;
- concurrent and historical negative control data with ranges, means and standard deviations;
- concurrent positive control (or non-concurrent DNA positive control) data;
- analytical determinations.

Discussion of the results:

Conclusion:

LITERATURE

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ANNEX

Definitions

Administration time: the total period during which an animal is dosed.

Base pair substitution: the type of mutation that causes the replacement of a single base nucleotide with another nucleotide of DNA.

Clonal expansion: the production of many cells from a single mutant cell.

Colony-forming unit (cfu): a measure of viable bacterial or fungal numbers.

Deletion: a mutation in which one or more (sequential) nucleotides is lost by the genome.

Electroporation: the application of electric pulses to increase the permeability of cell membranes.

Extrabinomial variation: greater variability in repeat estimates of a population proportion than would be expected if the population had a binomial distribution.

Frameshift mutation: a genetic mutation caused by insertions or deletions of a number of nucleotides that is not evenly divisible by three within a DNA sequence.

Insertion: the addition of one or more nucleotide base pairs into a DNA sequence.

Jackpot: a large number of mutants that arose through clonal expansion from a single mutation.

Ligation: the covalent linking of two ends of DNA molecules using DNA ligase.

Mitogen: a chemical substance that encourages a cell to commence cell division, triggering mitosis (i.e. cell division)

Large deletions: deletions in the size of more than several kilobases are effectively detected with the Spi-selection and the lacZ plasmid assays

Neutral gene: a gene that is not affected by positive or negative selective pressures.

Packaging: the synthesis of infective phage particles from a preparation of phage capsid and tail proteins and a concatamer of phage DNA molecules. Commonly used to package DNA cloned onto a lambda vector (separated by cos sites) into infectious lambda particles.

Packaging efficiency: the efficiency with which packaged bacteriophage are recovered in host bacteria.

Plaque forming unit (pfu): a measure of viable bacteriophage numbers.

Point mutation: the general term for a mutation affecting only a small sequence of DNA including small insertions, deletions, and base pair substitutions.

Positive selection: a method that permits only mutants to survive.

Reporter gene: a gene whose mutant gene product is easily detected.

Sampling time: the period of time, prior to sacrifice, during which the agent is not administered and during which any unprocessed DNA lesions are fixed into stable mutations

Shuttle vector: a vector constructed so that it can propagate in two different host species; accordingly, DNA inserted into a shuttle vector can be tested or manipulated in two different cell types or two different organisms.

Transgenic: of, relating to, or being an organism whose genome has been altered by the transfer of a gene or genes from another species.