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DRAFT TEST GUIDELINE

In Vitro Carcinogenicity: Syrian Hamster Embryo (SHE) Cell Transformation Assay

INTRODUCTION

1. *In vitro* cell transformation refers to the induction of phenotypic alterations in cultured cells that are characteristic of tumorigenic cells (1*)* (2). Transformed cells with the characteristics of malignant cells have the ability to induce tumours in susceptible animals (3) (4) (5); this validated the use of phenotypic alterations *in vitro* as criteria for a carcinogenic potential *in vivo*.

2. Since DNA damage and mutation are known to be initiating events for carcinogenesis, several short-term *in vitro* and *in vivo* tests are commonly used to identify genotoxicants. However, many chemicals that may not be detected by the traditional genotoxicity studies induce tumors through non-genotoxic mechanisms. The term *non-genotoxic carcinogen* refers to a lack of direct chemical effect on DNA primary structure in the initiation of tumors; on the contrary, *genotoxic carcinogens* (or their metabolites) are defined as compounds able to initiate carcinogenesis through direct interaction with DNA. The *in vitro* cell transformation assays (CTAs) have a high sensitivity for detecting both genotoxic and non-genotoxic carcinogens (6).

3. The performance of the Syrian Hamster Embryo (SHE) cell transformation assay as a predictor of carcinogenic potential has been established on hundreds of chemical carcinogens and has been reviewed in a database summarized in the OECD Detailed Review Paper (DRP) 31 (7)(8). An ECVAM (European Centre for the Validation of Alternative Methods) study (9) that addressed the availability of standardized protocols, their transferability, within- and between-laboratories reproducibility, in combination with the results of the DRP31, supports the use of the *in vitro* CTA for the assessment of carcinogenic potential (6).

4. This Test Guideline (TG) provides an *in vitro* procedure of the SHE cell transformation assay at pH 6.7 or 7.0, which may be used for hazard identification of chemical carcinogens. The test method described refers to the recommended protocol for this assay, as specified in Maire *et al.* (10).

INITIAL CONSIDERATIONS AND LIMITATIONS

5. The SHE cells are normal diploid, metabolically and p53-competent primary cells, which retain the ability to biotransform xenobiotics (11)(12). Exposure to carcinogenic chemicals results in an increase of morphologically transformed (MT) colonies, which are characterised by disorganised growth patterns and considered as an early stage in the carcinogenic process. It has been shown that SHE cells can be morphologically transformed by treatment with genotoxic and non genotoxic carcinogens.

6. The mechanisms behind carcinogenicity are multiple and not fully understood. Mechanisms of cell transformation have been related to changes in the expression of genes involved in cell cycle control, proliferation and differentiation. Genomic and epigenomic expression changes may result from genotoxic and non-genotoxic events. Non-genotoxic carcinogens have been postulated to act via a number of mechanisms such as inhibition of gap junction intercellular communication, oxidative stress, increased mitogenesis, decreased apoptosis, interference with tubulin polymerization, inhibition of senescence through activation of telomerase, as well as other epigenetic and heritable changes (4)(13)(14)(15)(16).

7. Disturbances of gene expression and genomic stability through hyper- or hypomethylation of DNA, histone modifications and nucleosomal remodeling are epigenetic mechanisms considered as fundamental in triggering a carcinogenic process (17). Interference with signal transduction pathways, and binding to receptors involved in hormone-mediated processes, peroxisome proliferation, activation of proto-oncogenes or inhibition of tumor suppressor genes through genotoxic or non-genotoxic mechanisms may also explain disruption of cell homeostasis and cell cycle control.

8. The subjectivity involved in identifying morphologically transformed colonies could impair the performance of the assay. However, studies utilizing photo galleries have shown the assay to have acceptable reproducibility (18)(19). Infrared spectroscopy is being explored to provide an objective determination of transformed colonies (20). In addition, molecular tools such as gene expression changes promise to provide useful molecular markers for morphological transformation, like those associated with cytoskeleton effects in the SHE cells (21).

9. The retrospective analysis of the SHE CTA results included in DRP31 covers 264 organic and inorganic chemicals[1]. In terms of prediction of human carcinogenicity, the SHE assay detected more than 90% of compounds classified as IARC group 1 (known human carcinogens), and 80-90% classified as IARC group 2A (probable carcinogens) and as IARC group 2B (possible carcinogens) as estimated from data in the DRP31 (7).

10. The concordance with *in vivo* rodent carcinogenicity study data is higher for SHE CTA than for most genotoxicity tests used for regulatory purposes (Annex IV of DRP 31). The mean percentage of false positive is lower than the percentage for mammalian cells genotoxicity tests established on a set of common chemicals (Annex IV of DRP 31) (7)(8).

11. SHE CTA results are expected to be used as part of a testing strategy and/or in a weight of evidence approach. When employed in combination with other information such as genotoxicity data, structure-activity analysis and pharmaco/toxicokinetic information, CTAs can contribute to the assessment of carcinogenic potential (22). It may be particularly useful for chemicals for which *in vivo* testing is not allowed (e.g. regulation on cosmetics in the European Union [Regulation (EC) 1223/2009 of the European Parliament and of the Council of 30 November 2009 on cosmetic products]), is limited, or is only required for chemicals identified as genotoxic (23).

12. When compared to genotoxicity tests, CTAs have the advantage of being able to also detect non-genotoxic carcinogens. Therefore, *in vitro* CTAs are useful in hazard assessment for carcinogenicity of chemicals.

PRINCIPLE OF THE TEST METHOD

13. SHE cells are obtained from primary cultures of individual Syrian hamster embryos at 13 days of gestation. After enzymatic tissue digestion, cells are collected and stored in liquid nitrogen. One part of cryopreserved SHE cells are used as feeder cells, the other part as target cells. The feeder

[1] Approximately: Industrial chemicals 50%, pharmaceuticals 25%, pesticides 11%, natural toxins 7%, food additive and dyes 7%,

cells will be x-ray irradiated to inactivate their capability to replicate, and seeded as nutrient base to support metabolic activity. The target cells are used to assess morphological transformation of colonies.

14. SHE cells (target cells) are seeded at clonal density onto a feeder layer of X-irradiated cells in culture conditions allowing for the development of colonies, and achieving the necessary cloning efficiency for fulfilling the acceptability criteria for the CTA (paragraph 52). After plating the cells, they are exposed to the test substance for 7 days. Thereafter, cells are washed, fixed and stained. Dishes are coded and colonies are scored for their morphological phenotype by *stereomicroscopy*.

15. Cytotoxicity is evaluated by inhibition of cloning efficiency and reduction in size/density of the colonies. The number of MT colonies relative to the total number of scorable colonies is calculated for each concentration tested. The frequency of morphologically transformed colonies relative to total number of colonies in the test substance-treated groups is compared to the frequency of morphologically transformed colonies in the appropriate control group.

PROCEDURE

Culture media, reagents and solutions

16. The culture medium, reagent and solutions used for cell preparation are described in Annex 1.

Culture conditions and Counting of viable cells

17. Cell cultures are incubated in a humidified incubator at 37°C and 10 ± 0.5 % CO_2. All centrifugation steps are carried out at 180-250 *g* for 10 minutes at 4°C. Viable cells are counted using the trypan blue dye exclusion test using 0.4% to 0.5% (w/v) trypan blue in buffered saline.

Preparation and cryopreservation of SHE cell stocks

18. SHE cells are isolated from 13-days gestation embryos of pregnant healthy female(s) sacrificed by ethical methods following OECD recommendations (24). Embryos are washed, transferred into sterile culture dishes containing wash solution, and the differentiated organs (head, viscera, and limbs) are discarded from each embryo. Embryos can be treated separately or pooled for tissue dissociation.

19. The remaining tissues are minced and dissociated by enzymatic digestion in dissociation solution under gentle stirring for 10 min at room temperature or at 37°C. The first lysate is discarded, and the dissociation is repeated 2-4 times. Cell suspensions are collected, centrifuged (at 4°C) and resuspended in cell isolation medium (CIM). Viable cells are counted and seeded (2 x10^6 /100 mm diameter culture dish, or 0.133 x 10^6 cells/cm^2 area of 150 or 225 cm^2 culture flasks) in CIM and incubated (37°C and 10 ± 0.5 % CO_2) until 60-80% cell growth confluency is achieved (usually within 24 to 48 hours). Then, cells are rinsed with buffered saline, detached with the appropriate detachment solution, and collected by centrifugation.

20. The cell pellet is suspended in CIM, viable cells are counted and pelleted by centrifugation. Cells are resuspended in cryopreservation medium, dispensed into storage vials (1.0 or 2.0 x 10^6 cells/vial), step frozen (i.e., successively for 30 minutes at 4°C, 4 hours at -20°C and one night at -80°C), and kept frozen under liquid nitrogen until use.

Checking of the SHE cells/FBS suitability

21. Before use, each new cell batch should be checked for spontaneous transformation rate, plating efficiency (colony forming ability) and morphological transformation using a positive control

substance. Likewise, any new batch of fetal bovine serum (FBS) should be checked for suitability. The combination "cell batch/FBS batch" should fulfil the acceptability criteria described in paragraph 53.

Preparation of feeder (irradiated) SHE cells

22. Cryopreserved SHE cells in frozen vials are thawed at 37°C, pelleted by centrifugation and resuspended in 10 mL fresh cell growth medium (CGM). This also eliminates most of the dimethylsulfoxide (DMSO) used in the cryopreservation medium. The cells are transferred to 100 mm culture dishes/flasks (2.0×10^6 cells/each dish or in T225 culture flask) and cultured in a humidified incubator at 37°C and 10 ± 0.5 % CO_2 for 2-4 days to achieve 50-90% confluency.

23. On the day of x-ray irradiation, cells are rinsed, detached and immediately resuspended in CGM. Cells are exposed to irradiation (5000 rads or 50 grays) so that they remain viable, but yet no longer capable of replication.

24. These freshly irradiated cells can be directly used for the experiments soon after irradiation. In case of cryopreservation of the irradiated cells, cells are centrifuged and the supernatant is removed. The pellet is resuspended in an appropriate volume of cold (hold on wet ice) cryopreservation medium. The viable cells are counted and dispensed into storage vials (5×10^6 cells/vials) on wet ice, and step frozen (as described in paragraph 20) prior to being stored frozen under liquid nitrogen. The cryopreservation step is a good way of keeping irradiated cells for longer period if an x-ray machine is not readily available.

Preparation of test solutions

25. The solutions of the test substance are prepared on the day of treatment. A series of solutions at different concentrations of the test substance (500 x the final concentration) in an appropriate vehicle are prepared under UV filtered lights or protected from light.

26. Concentrated solutions (500 x) of the test substance are diluted with the CGM to prepare ultimate dosing solutions at a concentration 2 x, so as to obtain the final concentration (1 x) in the test medium after addition of an equal volume of the test medium (Table 1, paragraph 32). If the test substance is not water soluble, a vehicle solvent, such as DMSO, is used. The final concentration should be the same in all vehicle control and treated dishes. In case DMSO is used as vehicle, the final concentration should not exceed 0.2%.

27. The maximum concentrations to be tested in cell transformation assay depend on solubility and cytotoxicity. For water soluble test substances, the highest dose level that can be tested for cytotoxicity is 5 mg/ml or 10 mM (paragraph 34). For non water soluble test substances, the highest dose tested is limited to the lowest precipitating dose in GCM (paragraph 34). For test materials insoluble at any concentration, suspensions may be tested up to high concentrations so as to obtain cytotoxicity.

Table 1: Concentrations of the test substance in the intermediate solutions and in the final medium

	Test substance dissolved in		
	Vehicle*	Dosing solution(4 mL test medium)	Final test medium (8 ml)
Concentration of the test substance	500 x	2 x	1 x
Concentration of the vehicle	100%	0.4%	0.2%

*For water insoluble test substances, concentrated solutions (500 x) may be prepared in DMSO

Experimental Design

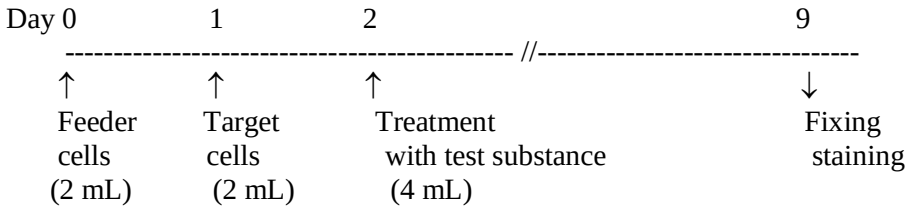

Figure 1: Timeline of the SHE CTA assay (the volumes are per each 60 mm culture dish)

Preparation of test cultures

Feeder layer

28. On day 0 (feeder cells day), the irradiated SHE cells are seeded. The cell concentration is adjusted to 20,000 – 30,000 cells/mL in CGM, and 2 mL are added into each 60 mm culture dish (4 to 6 x 10^4 feeder cells/dish). In case of cryopreservation of irradiated cells, cryopreserved cells are thawed at 37°C, and pelleted by centrifugation. The cell pellet is re-suspended in fresh CGM and the viable cells are counted. Freshly irradiated cells can also be used for seeding of the feeder layer.

29. The culture dishes are incubated in a humified incubator at 37°C and 10 ± 0.5% CO_2 for 24 hours before adding the target cells. For each test, at least 5 dishes filled with feeder cells only will be used concurrently as controls for the inability of the feeder cells to replicate and to form colonies. No colony is allowed to form in these dishes.

Target cells

30. Cryopreserved SHE cells are thawed at 37°C and seeded for growth in culture flasks. After an incubation period of either 5 or 24 hours, the target cells are detached, counted and the cell concentration is adjusted with CGM to a concentration where approximately 25 - 45 colonies/dish can be obtained at the end of the test (see paragraph 53). Two mL of the target cell suspension will be added to each culture dish containing feeder cells. Dishes will be incubated in a humidified incubator at 37°C and 10 ± 0.5% CO_2 for 24 hours prior to treatment with the test and control substances.

31. At cytotoxic dose level, as determined in the Dose Range Finding (DRF) experiment, the target cell number should be adjusted in order to obtain the recommended number of 25 to 45 colonies per at the end of the test to fulfil acceptance criteria (see paragraph 53). The adjustement of target cell number is explained in paragraph 36.

Treatment of cultures

32. For practicability, each dosing solution are prepared at a concentration twice (2 x) the final concentration expected (Table 1). Each dosing solution (4 mL) will be transferred to culture dishes (60 mm) already containing the CGM (4 mL) with feeder and target cells (final volume 8 mL) (Table 1). The cultures will be incubated in a humidified incubator at 37°C and 10 ± 0.5% CO_2 for 7 days without disturbance.

Preliminary Cytotoxicity / Dose Range Finding Assay

33. The maximum dose of the test substance will be determined taking into account the solubility and any relevant cytotoxicity information available for the test substance. A range of at least 10

concentrations to achieve a wide toxicity range will be tested in parallel to the vehicle control. At least five, preferably ten dishes will be seeded per concentration tested. The number of target cells seeded is the same in all dose groups. The conditions of testing (test medium, incubation conditions and time) are the ones described for the main experiment for cell transformation (see paragraphs 36-37,-39).

34. The highest dose level tested for soluble test substances is 5 mg/mL or 10 mM whichever is lower. Insoluble chemicals are tested up to the first concentration producing a visible opacity in the final test medium observable by the unaided eye. In all cases, the test substance solutions are prepared on the day of treatment. The cultures will be incubated in a humidified incubator at 37°C and 10± 0.5% CO_2 for a period of 7 days to allow colony development. The relative cytotoxicity of each treatment group will be measured by the reduction in plating efficiency and/or colony density and size of the treated SHE cells compared to the vehicle control.

Selection of Treatment Concentrations

35. Definitive assay doses are deduced from the dose range finding study and should include:

- A high test substance concentration causing a maximum 50% decrease of cytotoxicity axpressed by decrease in relative plating efficiency and/orreduction in relative colonies density/size (by visual appearance). If the test substance does not show a cytotoxic effect, the highest dose is either 5 mg/ml or 10 mM for a soluble compound, or the visible solubility limit in GCM for a non soluble compound;

- At least one concentration which has no apparent effect on plating efficiency;

- 3 or 4 intermediary concentrations.

Description of the Cell Transformation Assay

36. A sufficient number of target cells (around 150 cells/dish) to produce an average of 25 - 45 colonies at the end of the test will be dispensed in 2 mL of complete medium per 60 mm culture dish, each of which was seeded approximately 24 hours earlier with 4-6 x 10^4 feeder cells in 2 mL of GCM For cytotoxic concentrations, the number of target cells seeded should be increased so as to obtain the average number of colonies required (paragraph 53). For instance, an approximate 30 % reduction in the number of colonies may require to adjust the number of target cells to 1.5 x the number of cells seeded in the cytotoxicity assay; an approximate 50 % reduction, would lead to seed twice (2 x) the number of target cells seeded for cytotoxicity.

37. Twenty four hours after the seeding of the target cells, test and control substance treatment will be applied by addition to the test media (4 mL) of the appropriate dosing solution (4 mL) so as to obtain the final concentration required (Table 1).

38. At least 1000 colonies per treatment group (total of 40 dishes) are needed to establish a negative result in a transformation assay. The assay should include at least 5 different scorable concentrations of the test compound and the appropriate vehicle and positive controls. The treated cell cultures should be incubated for a period of 7 days in a humidified incubator (37°C, 10 ± 0.5 % CO_2) following treatment initiation. The culture dishes should be labelled as appropriate for identification.

39. After the incubation period of 7 days, the medium should be discarded from the dishes by aspiration, and the cells on the dishes should be rinsed with buffered saline. After removal of buffered saline, cells should be covered with fixing solution (ethanol or methanol) and let stand for at least 10 minutes in room temperature. The fixative is removed and the dishes are stained for approximately 20

minutes with 3-5 mL Giemsa solution. The stain is poured off and the plates are rinsed under tap water before the stained colonies are air-dried.

Morphological Cell Transformation

40. All dishes, including those of positive and negative controls, should be independently coded before microscopic analysis. The stained colonies are blindly scored under stereomicroscope for plating efficiency (PE) and morphological transformation (MT). Morphologically transformed colonies are characterized by a multi-layered, criss cross pattern of growth throughout the colony and piling up of cells. Individual cells within the colony are more basophilic relative to their normal counterparts, and have a decreased cytoplasm-to-nucleus ratio. Pictures of normal and morphologically-transformed colonies obtained at pH 6.7 and 7.0 can be found in the photo catalogue of Bohnenberger et al. (17) and Maire et al. (18), respectively.

41. Sparse colonies are not scored for MT, however, they are included in the total number of colonies for plating efficiency determination. If a colony contains less than 50 cells, it is not counted. Colonies at the edge of the plates can be scored for MT if clearly morphologically transformed.
Generally, for each concentration level, i.e. each test group, ≥ 1000 colonies are evaluated for morphological cell transformation (MT).

42. For each test group, normal (non-transformed) colonies and transformed colonies will be enumerated to evaluate the PE, RPE and MTF criteria detailed in paragraphs 50-52.

Controls

Vehicle control

43. In case the test substance is not water soluble, an appropriate vehicle control should be used. If DMSO is selected, it should be used at a concentration that will not exceed 0.2%. The final concentration of DMSO should be the same in all vehicle control and treated dishes : 0.2 %.

Positive control

44. Benzo[a]pyrene (B[a]P) may be used at the recommended concentrations of 1.0 or 5.0 µg/mL to demonstrate the sensitivity of the assay at pH 7.0 and pH 6.7 respectively. B[a]P should be dissolved in DMSO.

Feeder cells control

45. For each test, at least 5 dishes filled with feeder cells only should be used concurrently to confirm the inability of these cells to replicate and to form colonies. There should be no colony formed in these dishes at the end of the test period.

Solubility, pH, and Osmolality

46. The solubility of the test substance in the vehicle and in the test culture (medium) should be observed and documented.

47. The pH of the test substance dosing solutions should be measured after at least four hours of undisturbed incubation in an incubator, in humidified atmosphere at 37°C and 10 ± 0.5 % CO_2. Prior to performing the preliminary cytotoxicity assay, the test substance is dissolved in an appropriate solvent and diluted in GCM at a concentration equal or greater than the highest concentration to be tested. The pH of the treatment medium should be determined at the time of preparation and after at least 4 hours of incubation.

48. The osmolality of the treatment medium should be measured prior to or at the time of performing the preliminary cytotoxicity determination or the main experiment.

EVALUATION CRITERIA AND REPORTING

Morphological transformation

49. The morphological transformation frequency (MTF) should be calculated for each concentration level, i.e. each treatment group, using the data of one trial or of several trials pooled for each concentration level, as follows:

$$MTF = \frac{\text{number of transformed colonies}}{\text{total number of scorable colonies}} \times 100$$

Cytotoxicity

50. The average number of colonies per dish, the plating efficiency (PE) and the relative plating efficiency (RCE) should be determined for each test group.

51. The plating efficiency (% PE) and the relative plating efficiency (% RPE) will be calculated as follows:

$$PE = \frac{\text{total number of colonies per dish}}{\text{total number of target cells seeded per dish}} \times 100$$

$$RPE = \frac{\text{PE of dose group}}{\text{PE of the vehicle control group}} \times 100$$

52. In addition to the RPE, the colony size and density will be recorded as parameters of cytotoxicity. The size and density is observed and recorded as three categories:

- Normal (+)
- Slightly reduced (++ ; 20 – 30 % reduction)
- Greatly reduced (+++ ; 40 – 60 % reduction)

Acceptability Criteria and historical controls

53. The following criteria have to be fulfilled for the validity of the assay:

- 1000 colonies per treatment group should be available for morphological transformation (less than 1000 colonies are acceptable in case of significant increase in morphological transformation rate). However the average number of colonies per plate should normally not be less than 25.

- An average of 25-45 colonies per dish for each treatment group. A colony number beyond these limits is acceptable in the following cases: if MT results are negative, an average of less than 25 colonies per dish is acceptable; if MT results are positive, more than 45 colonies per dish are acceptable.

- Cloning efficiency of the negative/vehicle control is ≥ 20%.

- No colony formation is observed in the feeder cell control dishes. Feeder cells should be visible in the chemical treatment groups except if they are affected selectively by the test substance. If the feeder cells are affected by the test substance, then this observation should be recorded and reported.

- Transformation frequency in the negative controls (untreated and vehicle) within historical controls: based on historical data and data from the ECVAM validation study, the upper limit of transformation frequency in the negative controls (untreated and vehicle) is 0.6%.

- Increase in morphological transformation frequency of the positive control: the positive control substance should lead to a statistically significant increase in the morphological cell transformation compared to the vehicle control.

- To conclude to a negative result, four test substance treatment concentrations at least should be scorable and fulfilling the acceptability criteria.

Data interpretation criteria

54. Both statistical and biological relevant data are considered in the interpretation of the results, especially the level of concentration(s) increasing the MTF, regarding (i) effects at non-cytotoxic rather than cytotoxic concentrations, at environmentally or occupationally-relevant concentrations, (ii) coincidence with biological concentrations in human sera when data are available.

55. Results are analysed using the one-sided Fisher's exact test to determine if an increase in morphological transformation occurred at each concentration level compared to the concurrent vehicle control. A $p<0.05$ level of significance indicates a treatment related effect on MTF. When only one chemical concentration shows a statistically significant response, the Cochran-Armitage trend test for a positive dose-related response is performed.

56. A test chemical is considered **negative/non transforming**, if the percentage of morphologically transformed colonies in the test substance treated group is not statistically significant relative to the concurrent vehicle control (one-sided Fisher's exact test), or is ≤ 0.6%.

57. A test chemical is considered **positive** if it causes a significant increase in morphological transformation frequency (above 0.6%) either at two dose levels compared to the concurrent vehicle control (one-sided Fisher's exact test), or if one concentration shows a statistically significant increase and the Cochran-Armitage trend test is significant .

58. When results do not meet the criteria for a clear positive or a clear negative call (inconclusive results), the experiment should be repeated.

Test Report

59. The test report should include the following information:

Test substance
- identification data and CAS No. (if known)
- physical nature and purity

- physical properties relevant for conducting the assay
- stability of the test substance (if known)

Solvent/Vehicle (if appropriate)
- justification for choice of vehicle/solvent
- concentrations tested and preparation of the dosing solutions
- signs of precipitation (if appropriate)

Cells
- source of cells
- number of cell subcultures
- maintenance of cell cultures
- absence of mycoplasms

Test conditions
- rationale for selection of concentrations, including cytotoxicity data and solubility limitations
- composition of the media, CO_2, pH
- serum concentration, origin and quality
- concentrations of test substances
- volume of vehicle and test substance added
- duration of treatment
- incubation temperature
- number of cells plated
- positive and negative controls
- criteria for scoring MT colonies

Results
- cytotoxicity results
- pH, osmolality of culture media after addition of the test substance
- number of total scorable colonies
- relative cloning efficiency
- dose-response relationship, where possible
- statistical analyses
- concurrent negative (solvent/vehicle) and positive control data
- historical negative control data

Data should be presented in tabular form. The following values should be presented for each group (treated and untreated groups, vehicle and positive controls):

i. total number of colonies and of scorable colonies for each group
ii. relative plating efficiency %
iii. colony size/density
iv. number of transformed colonies
v. morphological transformation frequency (MTF %)
vi. Fisher's exact test p-value (one-sided)

Discussion of the results

Conclusion

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Annex 1: Culture medium, reagent and solutions used for cell preparation

The culture medium is DMEM: Dulbecco's Modified Eagle's Medium containing 1g/L glucose, 4 mM glutamine and 110 mg/L sodium pyruvate, with or without phenol red.

Depending on the pH selected, the DMEM medium is adjusted to pH 7.0 with 1.5 g/L $NaHCO_3$ or to pH 6.7 with 0.75 g/L $NaHCO_3$ and sterilized by membrane filtration (0.1 µm porosity). The culture medium can be stored at 4° C during a period not exceeding 2 weeks.
This culture medium serves to prepare the following media:

- Complete growth medium (CGM)

The complete culture medium is prepared with addition of fetal bovine serum (FBS) at a concentration of 15% or 20% (v/v) for the SHE pH 7.0 and the SHE pH 6.7 CTAs, respectively.

- Cryopreservation medium

The cryopreservation medium is the pH-adjusted DMEM, added with 10% FBS and 10% DMSO (or with 20% FBS and 7.5% DMSO).

- Cell isolation medium (CIM)

The cell isolation medium is the pH-adjusted DMEM added with 15% FBS and 1% antibiotics (penicillin 5000 U/mL; streptomycin 5000 µg/mL).

The solutions used for cell preparation and assay protocol are as follows:

- Buffered saline (*e.g.* calcium- and magnesium-free Hank's balanced solution (CMF-150 HBSS) or calcium- and magnesium-free phosphate buffered saline (CMF-PBS))

- Colony staining solution: 10% (v/v) Giemsa in ultra pure water

- Cell staining solution (*e.g.* 0.5% (w/v) trypan blue in buffered saline)

- Fixing solution: ethanol or methanol

- Detachment solution (*e.g.* 0.25% (w/v) trypsin in buffered saline or [0.05% (w/v) trypsin + 0.02% (w/v) $Na_2EDTA\text{-}H_2O$] in buffered saline)

- Dissociation solution (*e.g.* dispase 2 U/mL in buffered saline or [1.25% (v/v) Enzar-T, 2.5% (v/v) pancreatin + 2% (v/v) of penicillin 10,000 U/mL and streptomycin 10,000 160 µg/mL solution] in buffered saline)

- Wash solution: buffered saline with 1% (v/v) of penicillin 5,000 U/mL and streptomycin 5,000 µg/mL solution