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GUIDANCE DOCUMENT ON THE IN VITRO SYRIAN HAMSTER EMBRYO (SHE) CELL
TRANSFORMATION ASSAY

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FOREWORD

This document presents guidance for conducting the Syrian Hamster Embryo Cells Transformation Assay (SHE CTA).

This document was preceded by the development of the Detailed Review Paper (DRP) 31 on “Cell Transformation Assays for Detection of Chemical Carcinogens” (OECD, 2007), pre-validation study led by ECVAM, ESAC peer review, and then from 2011 by work aimed at the development of a Test Guideline. Despite support from some countries, concerns were expressed by others regarding the SHE CTA and the approval of the draft TG at the April 2013 WNT meeting was considered premature. Efforts were undertaken to try to address remaining scientific, technical issues, but nevertheless the draft Test Guideline did not reach the stage of regulatory acceptance.

In November 2014, the Joint Meeting discussed options for moving forward in the area of non-genotoxic carcinogenicity under the Test Guidelines Programme. The Joint Meeting advised 1) to proceed with the development of guidance documents on the SHE CTA and Bhas-42 CTA, mainly to describe the test procedures, and 2) to develop a guidance document at the OECD level outlining a conceptual framework for the identification of non-genotoxic carcinogens for priority setting.

The draft document has been through two WNT commenting rounds in July and in December 2014. Since all countries who commented either indicated approval or indicated that their comments did not impede approval of the document and were only of editorial nature, this document was approved by written procedure.

This document is published under the responsibility of the Joint Meeting of the Chemicals Committee and the Working Party on Chemicals, Pesticides and Biotechnology.
GUIDANCE DOCUMENT ON THE
IN VITRO SYRIAN HAMSTER EMBRYO (SHE) CELL TRANSFORMATION ASSAY

PURPOSE

1. The purpose of this Guidance document is to allow the regulatory community to use the described method as part of a weight of evidence approach in the testing of substances for carcinogenic potential. There are a number of issues which have impeded consensus on the approval of the Test Guideline; these issues mainly include the subjective nature of evaluating transformed phenotypic morphology, the limited understanding of causal molecular mechanisms leading to the transformed SHE colonies, the relatively small number of bona fide non-genotoxic carcinogens, as compared to genotoxic carcinogens, that have been tested in the SHE cell transformation assay, and the way the assay might be used in a regulatory framework.

Background

2. In vitro cell transformation refers to the induction of phenotypic alterations in cultured cells. Cell transformation is an event in the multi-step process of tumour induction (1) (2). Transformed cells are phenotypically different from normal cells and have the ability to induce tumours in susceptible animals (3) (4) (5). It has been shown that SHE cells can be morphologically transformed by treatment with genotoxic and non genotoxic carcinogens (6) (7) (8). Exposure results in an increase of morphologically transformed (MT) colonies, which are characterised by disorganised growth patterns and mimicking an early stage in the carcinogenic process.

3. The performance of the Syrian Hamster Embryo (SHE) cell transformation assay conducted at a variety of pHs to detect transforming activity has been established on a large set of substances and has been reviewed in a database summarized in the OECD Detailed Review Paper (DRP) 31 (6) (9). In addition, the European Reference Laboratory for Alternatives to Animal Testing (EURL ECVAM) study (10) (11) addressed the availability of standardized protocols, their transferability, within- and between- laboratories reproducibility. The EURL ECVAM work also included the analysis of the degree of similarity between protocols. It concluded that there are no elements suggesting that the EURL ECVAM experiments differ notably from the experiments reported in the OECD DRP 31, thus making these data acceptable for use in a retrospective evaluation.

4. When SHE CTA results are used as part of a testing strategy (not as results from a stand-alone assay) and/or in a weight of evidence approach, they may contribute to the assessment of carcinogenic potential of test chemicals (12). While the available data (see paragraph 14) would suggest that the SHE CTA has greater sensitivity for carcinogens acting via genotoxic mechanisms, for non-genotoxic carcinogens the added value of the SHE CTA is still debated and more information is required.

5. This Guidance Document (GD) provides an in vitro procedure of the SHE cell transformation assay, as specified in Maire et al. (13) or in the EURL ECVAM DB-ALM protocol on SHE CTA (14), conducted at pH 6.7 and 7.0. The assay can be performed at either pH 6.7 or 7.0 (see paragraphs 14) provided proficiency has been demonstrated at the chosen pH (see paragraph 54-55). The morphology of the normal colonies differs slightly at physiological pH compared to acidic pH, however, the conduct of the assay at either pH has been shown to give similar results. Other than the difference in the pH, the experimental protocol for both versions of the assay is the same.
Current knowledge and understanding about mechanisms involved in cell transformation/

6. The exact molecular mechanisms involved in cell transformations are only partially understood (15) (16) (17). Although there are uncertainties regarding the causal mechanisms leading to the transformed SHE colonies, the following paragraphs review current knowledge and understanding based on the literature.

7. Evidence indicates that cell transformation results from alterations and changes in the expression of genes involved in cell cycle control, genomic stability, proliferation and differentiation. Genetic changes affecting these processes may result from direct genotoxic mechanisms. Also, disturbances of gene expression and genomic stability through hyper- or hypomethylation of DNA, histone modifications and nucleosomal remodelling are epigenetic mechanisms considered as fundamental in triggering a carcinogenic process (18). Consistent with these diverse mechanisms, some SHE cell transformants have been shown to harbour biallelic, inactivated p53 tumour suppressor genes (19). Carcinogens such as DES can suppress DNA methylation in short-term treatments (20). The initial transformants induced by polycyclic aromatic hydrocarbons frequently display DNA methylation-associated suppression of gene expression known to be associated with embryonic differentiation and engineered re-expression suppresses the transformed phenotype (21). SHE cell transformation by diethanolamine is driven by altered choline metabolism, an important methyl donor in one-carbon metabolism leading to DNA methylation (22). Introduction of an activated oncogene (v-Ha-ras), by transfection, will morphologically transform normal SHE cells (23). Increased frequency of akinetochoric chromosome disjunction occurs during the growth of the initial transformants (24) which could contribute to the aneuploid characteristic of immortalized clones arising from such populations (reviewed in Ahmadzai et al., 2012 (25)).

8. Among later stage immortal and malignantly transformed descendents, global DNA hypomethylation and site specific hypomethylation in ras and myc oncogenes have been observed (20) (26). Also, methylation-associated suppression of cell cycle checkpoint gene expression, or mono- or biallelic losses of these genes (ink4a, ink4b), as well as mutations in p53 have been found in immortal Syrian hamster dermal cells (27). In morphologically transformed SHE cell lines, cell cycle checkpoint control (G2) is often compromised (28). An activated proto-oncogene (cph) capable of transforming other cells has been isolated from malignantly transformed SHE cells (29).

9. 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, interference with signal transduction pathways, and binding to receptors involved in hormone-mediated processes, and in peroxisome proliferation. Instances of several of these mechanisms have been demonstrated in SHE cell transformation. Oxidative stress was shown to be causally involved in morphological transformation (30) (31). Imbalance of cell proliferation via an inhibition of apoptosis has been related to cell transforming effects of some hepatic peroxisome proliferators and other transforming agents in SHE cells (32) (33). Growth factor treatments of SHE cells, presumably acting through signal transduction pathways, can also drive transformation (34) and gap junctional cell-to-cell communication is frequently impaired by non-genotoxic carcinogens (35).

INITIAL CONSIDERATIONS AND LIMITATIONS

10. The SHE cells are normal diploid, metabolically and p53-competent primary cells, which retain the ability to biotransform a wide range of xenobiotics as evidenced by studies with substances requiring metabolic activation (6) (9) (36) (37) (38). From a 3Rs perspective, the use of primary cells means that a
small number of pregnant hamsters are euthanized; one hamster provides sufficient cells to perform at least 50 to 100 CTAs providing the cells are adequately preserved for future use. The metabolic capability of the cells should be considered and discussed in the light of interpretation of test results. This is particularly important when the test chemical requires metabolic activation. Exposure to test chemicals with transforming capacity results in an increased number of morphologically transformed (MT) colonies, which are characterised by disorganised growth patterns.

11. Transformation of primary, diploid SHE cells appears to follow a staged process. The transformation assay in the SHE cells is based upon identifiable colonies of morphologically transformed cells with irregular growth patterns. The transformants are thought to be stem cells with blockages in their differentiation pathways (39). Upon further passages in vitro, transformed colonies clonally isolated from treated cultures frequently generate cells with an infinite cellular lifespan or an ability to form tumours in syngenic hosts. Untransformed clones become senescent (40) (41) (42). High frequencies of progression to immortality and anchorage independence were also observed in bulk cultures of SHE cells (4).

12. Although conducted blindly, identification of morphologically transformed colonies by microscopic scoring is subjective, as for any cytohistochemical endpoint. This subjectivity may, to some extent, be overcome with appropriate training, and the use of photo catalogues (43) (44). However, to improve the reliability of the scoring a second opinion or duplicate independent scoring is highly recommended, especially for ambiguous colonies/or borderline pictures.

13. The assay would be improved by the development of objective measures for scoring transformation, when these are validated. Some examples include biospectroscopy, which is being explored to provide an objective determination of transformed colonies (45). 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 (38).

14. To date comparative sensitivity and specificity of the pH 6.7 and 7.0 versions of the assay are limited to a small chemical database (see annex to DRP 31(7)). An analysis of the sensitivity and specificity of the assay for genotoxic and non-genotoxic carcinogens that were fully tested in in vitro and in vivo genotoxicity assays was performed in 2014 and is available in an annex to the DRP.

15. When planning the experiment, careful choice of the optimum pH needs to be taken into account. Parameters might include ionisable nature of the compounds as affecting the differences in reactivity or bioavailability. The historical experience of the laboratory with the scoring at either pH should also be considered. This needs to be taken into account until a wider chemical database has been generated.

16. At this time, the assay conducted as described in this Guidance Document does not provide information on in vivo potency, or species-specificity or tissue-specificity of the cell transformations.

17. It should be noted that this method has been validated for mono-constituent substances only and not multi-constituent substances, UVCBs (substances of unknown or variable composition, complex reaction products or biological materials) or mixtures. Before use of the assay for the testing of a mixture intended for a regulatory purpose, it should be considered whether, and if so why, it may provide adequate results for that purpose. Such considerations are not needed, when there is a regulatory requirement for testing of the mixture.

PRINCIPLE OF THE TEST METHOD

18. SHE cells are obtained from primary cultures of Syrian hamster embryos at 13 days of gestation. After enzymatic tissue digestion, cells are collected, grown for 24 to 48 hours and then cryopreserved, and stored in liquid nitrogen. One part of cryopreserved SHE cells is used as feeder cells, the other part as
target cells. The feeder cells are x-ray irradiated to inactivate their capability to replicate, and seeded as nutrient base and support of metabolic activity. The target cells are used to assess morphological transformation of colonies.

19. SHE cells (target cells) are seeded at clonal density onto a feeder layer of x-ray-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 60). After plating the cells, they are exposed to the test chemical for 7 days. Thereafter, cells are washed, fixed and stained. Dishes are coded and colonies are scored for their morphological phenotype by stereomicroscopy.

20. Cytotoxicity is evaluated by inhibition of cloning efficiency and reduction in size/density of the colonies. The number of morphologically transformed (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 chemical-treated groups is compared to the frequency of MT colonies in the solvent control group.

DESCRIPTION OF THE METHOD

Preparations

Culture media, reagents and solutions

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

Culture conditions and counting of viable cells

22. Cell cultures are incubated in a humidified incubator at 37°C and 10 ± 0.5 % CO2. 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

23. SHE cells are isolated from 13-days gestation embryos of pregnant healthy female(s) humanely euthanized. Embryos are washed, transferred into sterile culture dishes containing wash solution, and the differentiated organs (head, viscera, and limbs) are discarded from each embryo. Cells can be prepared from single embryos, embryos pooled from a single dam or embryos pooled from different dams sacrificed at the same time.

24. The remainder of the embryo is minced and dissociated by enzymatic digestion in dissociation solution under gentle stirring for 10 min at room temperature or at 37°C. The first wash is discarded, and the dissociation is repeated 2-4 times. Cell suspensions are collected, centrifuged (at 4°C) and re-suspended in cell isolation medium (CIM). Viable cells are counted and seeded (2 x10⁶ /100 mm diameter culture dish, or 0.133 x 10⁶ cells/cm² area of 150 or 225 cm² culture flasks) in CIM and incubated (37°C and 10 ± 0.5 % CO₂) 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.

25. The cell pellet is suspended in CIM, viable cells are counted and pelleted by centrifugation. Cells are re-suspended in cryopreservation medium, dispensed into storage vials (1.0 or 2.0 x 10⁶ 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

26. 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 chemical. Likewise, any new batch of foetal bovine serum (FBS) should be checked for suitability. The combination “cell batch/FBS batch” should fulfil the acceptability criteria described in paragraph 60.

Preparation of feeder (irradiated) SHE cells

27. Cryopreserved SHE cells in frozen vials are thawed at 37°C, pelleted by centrifugation and re-suspended in fresh cell growth medium (CGM). This also eliminates most of the dimethylsulfoxide (DMSO) used in the cryopreservation medium. Ten or 40 mL aliquots of cells are transferred to respectively 100 mm culture dishes or T225 culture flasks (2.0 x 10^6 cells/dish or 8.0 x 10^6 cells in T225 culture flask) and cultured in a humidified incubator at 37°C and 10 ± 0.5 % CO₂ for 2-4 days to achieve 50-90% confluence.

28. On the day of x-ray irradiation, cells are rinsed, detached and immediately re-suspended in CGM. Cells are exposed to irradiation (5000 rads or 50 grays) so that they remain viable, but no longer capable of replication. Before and after irradiation, cells should be maintained on ice, and preferably also during irradiation.

29. 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 re-suspended in an appropriate volume of cold (hold on wet ice) cryopreservation medium. The viable cells are counted and dispensed into storage vials (5 x 10^6 cells/vial) on wet ice, and step frozen (as described in paragraph 25) prior to being stored frozen under liquid nitrogen. The cryopreservation step is a good way of keeping irradiated cells for a longer period if an x-ray machine is not readily available.

Preliminary cytotoxicity and dose range finding (DRF) assay

30. The maximum dose of the test chemical should be determined taking into account the solubility and any relevant cytotoxicity information available for the test chemical. In the DRF, a range of at least 10 concentrations to achieve a wide toxicity range should be tested in parallel to the solvent control. At least five, preferably ten dishes should be seeded per concentration tested. The number of target cells seeded is the same in all treatment groups. The conditions of testing (test medium, incubation conditions and time) are the ones described for the main experiment for cell transformation (see paragraphs 41-44).

Test conditions

Solvent use

31. The solvent should be chosen to optimize the solubility of the test chemical without adversely impacting the assay conduct, e.g. cell growth, integrity of the test material, reaction with culture vessels. It is recommended that, wherever possible, the use of an aqueous solvent should be considered first. Well established solvents are for example water, cell culture medium, and dimethyl sulfoxide. Generally the final concentration of organic solvents in the tissue culture medium should not exceed 0.2% (v/v). This may be achieved by diluting concentrated solutions (500 x) of the test chemical in 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 (see Table 1 and paragraph 33 as examples). If other than well-established solvents are used, their use should be supported by data indicating their compatibility with the test chemical, the test system, and their lack of transforming potency. In such cases, untreated controls should also be included.
Selection of test concentrations

32. The maximum concentrations to be tested in cell transformation assay depend on test chemical solubility and cytotoxicity. For test chemicals of defined composition the highest dose level should be 0.01 M, 2 mg/mL or 2 µL/mL, whichever is the lowest. For test chemical of non-defined composition, e.g. complex mixtures (plant extracts, tars, environmental extracts etc.), the top concentration should be at least 5 mg/mL. Poorly soluble chemicals should be tested up to the first concentration producing a visible opacity in the final test medium observable by the unaided eye.

33. In addition to the controls, at least 5 test chemical concentrations should be used in the main experiment. These are deducted from the range finding study and should include:

- A high test concentration inducing no more than 50% cytotoxicity expressed by decrease in relative plating efficiency and/or reduction in relative colonies density/size (by visual appearance). If the test chemical does not show a cytotoxic effect, the highest dose is selected as indicated in paragraph 32 above for soluble test chemicals, or as the visible solubility limit in the final test medium for insoluble test chemicals;
- At least one concentration which has no apparent effect on plating efficiency;
- 3 or 4 intermediate concentrations.

Table 1: Recommended concentrations of the test chemical in the intermediate solutions and in the final test medium

<table>
<thead>
<tr>
<th>Concentration of the test chemical</th>
<th>Solvent*</th>
<th>Intermediate solution (4 mL test medium)</th>
<th>Final test medium (8 mL = 4mL intermediate solution+4mL GCM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>500x</td>
<td>100%</td>
<td>2x</td>
<td>1x</td>
</tr>
<tr>
<td>Concentration of the solvent</td>
<td>100%</td>
<td>0.4%</td>
<td>0.2%</td>
</tr>
</tbody>
</table>

*For water insoluble test chemicals, concentrated solutions (500x) may be prepared in Dimethyl sulfoxide (DMSO). For water soluble test chemicals, an aqueous solvent is recommended.
Day 0                  1                   2                                                         9

--------------------------------------------//--------------------------------------------

Feeder         Target             Treatment                                          Fixing

cells                 cells                 with test chemical                            staining
(2 mL)           (2 mL)             (4 mL)

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

Preparation of test cultures

Feeder layer

34. 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 of the cell suspension are added into each 60 mm culture dish (4 to 6 x 10⁴ 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.

35. The culture dishes are incubated in a humified incubator at 37°C and 10 ± 0.5% CO₂ 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 should form in these dishes.

Target cells

36. Cryopreserved SHE cells are thawed at 37°C and seeded for growth in culture flasks. After an incubation period (usually of 24 hours although shorter duration e.g. 5 hours can be used), 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 60). 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% CO₂ for 24 hours prior to treatment with the test and control chemicals.

37. At cytotoxic dose levels, as determined in the Dose Range Finding (DRF) experiment, the target cell number should be adjusted in order to yield the recommended number of 25 to 45 colonies per dish at the end of the test to fulfil acceptance criteria (see paragraph 60). The adjustment of target cell number is explained in paragraph 37.

Treatment of cultures

38. For practicality, one way of preparing dosing solution is to start with a concentration representing twice (2x) the final concentration (Table 1). Each dosing solution (4 mL) will be transferred to individual 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% CO₂ for 7 days without disturbance.
PROCEDURE

Preparation of test solutions

39. The test chemical solutions are prepared on the day of treatment. Solid test chemicals should be dissolved in appropriate solvents and diluted, if appropriate, prior to treatment of the cells. Liquid test chemicals may be added directly to the final test medium and/or diluted prior to treatment. Gaseous or volatile chemicals should be tested by appropriate methods, determined on a case by case basis. Fresh preparations of the test chemical should be used unless stability data demonstrate the acceptability of storage. A series of solutions at different concentrations of the test chemical should be prepared under UV filtered lights or protected from light.

Description of the cell transformation assay

40. A sufficient number of target cells (around 150 cells/dish, but the number of target cells is dependent on the cell batch/FBS batch) 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 CGM. For cytotoxic concentrations, the number of target cells seeded should be increased so as to maintain the target range of 25-45 colonies per dish (paragraph 60). For instance, an approximate 30 % reduction in the number of colonies may require to adjust the number of target cells to 1.5x the number of cells seeded in the cytotoxicity assay; an approximate 50 % reduction, would lead to seed twice (2x) the number of target cells seeded for cytotoxicity.

41. Twenty four hours after the seeding of the target cells, test and control chemical treatment will be initiated by addition to the test media (4 mL) of the appropriate dosing solution (4 mL) so as to obtain the final concentration of 0.2% (Table 1).

42. The treated cell cultures should be incubated for a period of 7 days in a humidified incubator (37°C, 10 ± 0.5 % CO₂) following treatment initiation to allow colony development. The culture dishes should be labelled as appropriate for identification. The relative cytotoxicity of each treatment group should be measured by the reduction in plating efficiency and/or colony density and size of the treated SHE cells compared to the solvent control.

43. After the incubation period of 7 days, the medium should be discarded from the dishes by aspiration, and the cells attached to the dishes should be rinsed with buffered saline. After removal of the buffered saline, cells should be covered with fixing solution (ethanol or methanol) and kept for at least 10 minutes at 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 dishes are rinsed with tap water before the stained colonies are air-dried.

Morphological cell transformation

44. All dishes, including those of positive and negative controls, should be coded before microscopic analysis. The stained colonies are evaluated and scored under stereomicroscope for plating efficiency (PE) and morphological transformation (MT). The scorer should be unaware of the coding. Morphologically transformed colonies are characterized by a criss-cross pattern of growth 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, which can be found in the photo catalogue of Bohnenberger et al. (43) and Maire et al. (44), respectively, should be used routinely.
Sparse colonies are not scored for MT (i.e. if a colony contains less than 50 cells, it is not counted); however, they are included in the total number of colonies for plating efficiency determination. Colonies at the edge of the dishes should be scored for MT if clearly morphologically transformed. Generally, for each treatment group $\geq 1000$ colonies should be evaluated for morphologically cell transformation (MT).

For each treatment group, normal (non-transformed) colonies and transformed colonies will be enumerated to evaluate the plating efficiency (PE), the relative plating efficiency (RPE) and the morphological transformation frequency (MTF) criteria detailed in paragraphs 56-58.

Controls

Solvent control

In case the test chemical is not water soluble, an appropriate solvent control should be used. If DMSO or other organic solvents are selected, they should be used at a final concentration that does not exceed 0.2% (see paragraph 31). The final concentration of any solvent should be the same in all solvent control and treated dishes.

Positive control

Because of the high amount of data on Benzo[a]pyrene (B[a]P), it should be used as positive control at the recommended concentrations of 1.0 to 5.0 $\mu$g/mL in dimethyl sulfoxide (DMSO) to demonstrate the sensitivity of the assay. However, if justified, other chemicals can also be considered as positive controls. Each laboratory should establish the performance of the positive control under their own laboratory conditions (see paragraph 49).

Feeder cells control

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

Solubility, pH, and osmolality

The solubility/precipitation of the test chemical in the solvent and in the test culture (medium) should be visually assessed and documented in the test report.

The pH of the test chemical dosing solutions should be measured at the time of preparation of the treatment medium and after at least four hours of undisturbed incubation in an incubator, in humidified atmosphere at 37°C and 10 ± 0.5 % CO$_2$. In case of deviation from the expected pH at any time point, the pH of the medium should be adjusted to the selected pH. Any deviation should be reported and considered in the interpretation of the results.

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. Osmolality of the treatment medium should be compared to the control medium, and any change used in the interpretation of results.

Proficiency of the laboratory

In order to demonstrate proficiency, a laboratory should perform tests with the four positive chemicals acting via different mechanisms, and two negative chemicals, included in Table 2. Proficiency
should be demonstrated at the pH the assay will be conducted, i.e. pH 6.7 and/or pH 7.0. The choice of the negative chemicals will depend of the version of the assay the laboratory demonstrates proficiency with. During the course of these tests, the laboratory should establish:

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

54. Re-evaluation of laboratory proficiency is recommended if major changes to the experimental conditions are proposed for the assay (e.g. use of automated instead of manual scoring techniques). Before starting to use this method, it is recommended that personnel are trained in a laboratory experienced in the performance of this assay.

### Table 2: Substances for Assessing Laboratory Proficiency

<table>
<thead>
<tr>
<th>Category</th>
<th>Substance</th>
<th>CASRN</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Carcinogens(^1)</td>
<td>Benzo[a]pyrene</td>
<td>50-32-8</td>
</tr>
<tr>
<td></td>
<td>2,4-diaminotoluene</td>
<td>95-80-7</td>
</tr>
<tr>
<td></td>
<td>o-toluidine</td>
<td>636-21-5</td>
</tr>
<tr>
<td></td>
<td>Non-genotoxic carcinogen</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Di (2-Ethylhexyl)phthalate (DEHP)</td>
<td>117-81-7</td>
</tr>
<tr>
<td>2. Non carcinogens(^2)</td>
<td>Anthracene</td>
<td>120-12-7</td>
</tr>
<tr>
<td></td>
<td>d-maniol (only for pH 6.7)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>1 naphtylamine (only for pH 6.7)</td>
<td>69-65-8</td>
</tr>
<tr>
<td></td>
<td>Benzoin (only for pH 7.0)</td>
<td>119-53-9</td>
</tr>
</tbody>
</table>

\(^1\) Rodent carcinogen: sufficient evidence in animals.  
\(^2\) Rodent non-carcinogen: inadequate or no evidence in animals.

**DATA AND REPORTING**

*Morphological transformation*

55. The morphological transformation frequency (MTF) should be calculated for each treatment group, using the data of one trial, as follows:

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

*Cytotoxicity*

56. The average number of colonies per dish, the plating efficiency (PE) and the relative plating efficiency (RPE) should be determined for each treatment group.

57. The plating efficiency (% PE) and the relative plating efficiency (% RPE) should 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 treatment group}}{\text{PE of the solvent control group}} \times 100

58. In addition to the RPE, the colony size and density (number of cells per colony) should be recorded as parameters of cytotoxicity. The size and density is observed and recorded as three categories:

- Normal (+)
- Slightly reduced (++ ; 20 – 39 % reduction)
- Greatly reduced (+++; 40 – 60 % reduction)

**Acceptability Criteria and historical controls**

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

- At least 1000 colonies per experimental group should be available for morphological transformation scoring. Occasionally, in case of a significant increase in morphological transformation rate, less than 1000 colonies are acceptable. However the average number of colonies per dish should normally not be less than 25.
- An average of 25-45 colonies per dish should be available (46). Occasionally, in case of a negative result, dishes with less than 25 colonies per dish are acceptable. Likewise, in case of a positive result, dishes with more than 45 colonies are acceptable.
- Cloning efficiency of the negative/solvent control is \geq 20%.
- No colony formation should be observed in the feeder cell dishes. Feeder cells should be visible in the chemical treatment groups except if they are affected selectively by the test chemical. If the feeder cells are affected by the test chemical, then this observation should be recorded, reported, and considered in the interpretation of the results.
- Transformation frequency in the negative controls (untreated and solvent) is within the distribution of historical control data of the laboratory (e.g. 95% confidence interval). Based on historical data from experienced laboratories and data from the EURL ECVAM validation study, the upper limit of transformation frequency in the negative controls (untreated and solvent) is 0.6%.
- The positive control chemical should induce a biologically relevant and statistically significant increase in morphological cell transformation compared to the solvent control.

**Data interpretation criteria**

60. Although biological relevance of the results should be considered first, both statistical significance and biological relevance of data are considered in the interpretation of the negative and positive results. The level of concentration(s) increasing the MTF is carefully considered, taking into account the range of cytotoxic/non-cytotoxic concentrations.

61. Providing that all acceptability criteria are fulfilled, the following criteria are considered for the evaluation of results:
(1) the increase in MT colonies is concentration-related,
(2) at least one of the test concentration exhibits a statistically significant increase compared to the concurrent negative control,
(3) the statistically significant result is outside the distribution range of the historical negative control data (e.g. 95% confidence interval).

62. A result can be considered clearly biologically relevant and a test chemical is considered a clear positive if all the above criteria are met.

63. A test chemical is considered as a clear negative if none of the criteria above (paragraph 62) are met.

64. Results are statistically 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 solvent control. A p<0.05 level of significance indicates a treatment related effect on MTF. The Cochran-Armitage trend test can be used to contribute to the evaluation of positive concentration-related response.

65. When results do not meet the criteria for a clear positive or a clear negative call, the test chemical should be evaluated by expert judgement and/or the experiment should be repeated. Modification of study parameters over an extended or narrowed range of concentrations, as appropriate, should be considered in follow-up experiments. In rare cases, even after further investigations, the data set will preclude making a conclusion of positive or negative results, and will therefore be concluded as equivocal.

Test report

66. The test report should include the following information:

Test chemical

Mono-constituent substance:
- physical appearance, water solubility, and additional relevant physicochemical properties;
- chemical identification, such as IUPAC or CAS name, CAS number, SMILES or InChI code, structural formula, purity, chemical identity of impurities as appropriate and practically feasible, etc.

Multi-constituent substance, UVBCs and mixtures:
- characterised as far as possible by chemical identity (see above), quantitative occurrence and relevant physicochemical properties of the constituents.

Solvent (if appropriate)
- justification for choice of solvent
- concentrations tested and preparation of the dosing solutions
- signs of precipitation (absence or presence)

Cells
- source of cells
- number of cell subcultures
- maintenance of cell cultures
- absence of mycoplasms
- identification of serum (provider and batch number)

**Test conditions**
- rationale for selection of concentrations, including cytotoxicity data and solubility limitations
- composition of the media, CO₂, pH
- serum concentration, origin and quality
- concentrations of test chemicals
- volume of solvent and test chemical added
- duration of treatment
- incubation temperature
- number of cells plated
- positive and negative controls
- criteria for scoring MT colonies

**Results**
- cytotoxicity results
- signs of precipitation
- pH, osmolality of culture media after addition of the test chemical
- number of total scorable colonies
- relative cloning efficiency
- concurrent feeder cell control
- dose-response relationship, where possible
- statistical analyses
- concurrent negative (solvent) and positive control data
- historical negative (solvent) and positive control data, with ranges, means, standard deviation, and confidence interval (e.g. 95%)

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

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

**Discussion of the results**

**Conclusion**
REFERENCES


transformation of Syrian hamster embryo (SHE) cells by Malachite green, Cancer Letters, 237 (2), pp. 188-198.


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. The media can be purchased readymade from the vendors and should be stored according to the parameters (time, temperature) provided with the batch media.

If powder media is used, depending on the pH selected, the DMEM medium is adjusted to pH 7.0 with 1.5 g/L NaHCO₃ or to pH 6.7 with 0.75 g/L NaHCO₃ 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 (recommended if the test is carried out at pH 6.7).

- **Cell isolation medium** (CIM)
  The cell isolation medium is the pH-adjusted DMEM added with 15% FBS and antibiotics penicillin 100 U/mL and streptomycin 100 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- HBSS) or calcium- and magnesium-free phosphate buffered saline (CMF-PBS))

- **Colony staining solution**: 10% (v/v) Giemsa in aqueous buffer

- **Cell staining solution** (*e.g.* 0.4% to 0.5% (w/v) trypa 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) Na₂EDTA-H₂O] 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 with 200 U/mL of penicillin and 200 µg/mL streptomycin] in buffered saline)

- **Wash solution**: buffered saline with 200 U/mL of penicillin and 200 µg/mL streptomycin