February 2013

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 validates 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 tumours; 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 (7) without discrimination between the two. Discrimination can be accomplished however, if used in parallel with genotoxicity test(s).

3. The performance of the Syrian Hamster Embryo (SHE) cell transformation assay conducted at a variety of pHs 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). A European Centre for the Validation of Alternative Methods (ECVAM) 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). The ECVAM work (9) also included the analysis of the degree of similarity between protocols. It concluded that there are no elements suggesting that the ECVAM experiments stand out from the experiments reported in the OECD DRP 31, thus making these data acceptable for use in a retrospective evaluation. A more recent analysis based on the limited number of chemicals available to date and tested at both pH 6.7 and 7.0, indicates that both versions of the assay currently perform with relatively high sensitivity and specificity (annex of DRP 31 (7)).

4. SHE CTA results are expected to be used as part of a testing strategy (not as results from a stand-alone assay) 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) and may reduce the use of *in vivo* testing. 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).

5. This Test Guideline (TG) provides an *in vitro* procedure of the SHE cell transformation assay 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) or in the EURL ECVAM DB-ALM protocol on SHE CTA (28), which was conducted at 6.7 and 7.0.

6. In this Test Guideline, the test can be performed at either pH 6.7 or 7.0 (see paragraph 13). The morphology of the control colonies differs at physiological pH compared to acidic pH, however, the conduction of the assay at either pH has been shown to give similar results in terms of concordance with rodent bioassay data. Other than the difference in the pH, the experimental protocol for both versions of the assay is the same.

INITIAL CONSIDERATIONS AND LIMITATIONS

7. 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 carcinogens requiring metabolic activation (7) (8) (11) (12). From a 3Rs perspective, the use of primary cells means that a small number of pregnant hamsters are euthanized; however, one hamster provides sufficient cells to perform at least 50 to 100 CTAs providing the cells are adequately preserved for future use. Exposure to carcinogenic chemicals 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. It has been shown that SHE cells can be morphologically transformed by treatment with genotoxic and non genotoxic carcinogens (7) (26) (27).

8. The mechanisms behind rodent and human carcinogenicity are multiple and not always fully understood. Likewise, the exact molecular mechanisms involved in cell transformations are only partially understood (14) (15) 16). Transformation of primary, diploid SHE cells is considered a model of the multistep process of carcinogenesis, as it appears to follow a staged process. The transformation assay in the SHE cells is based upon products of the initial phase of the process, which are the identifiable colonies of morphologically transformed cells with deranged growth patterns. The transformants are thought to be stem cells with blockages in their differentiation pathways (LeBoeuf et al., 1999). The transformed phenotype is also characterized as a neoplastic progression-predisposing state that permits further steps toward acquisition of immortality, tumourigenicity and, finally, full malignancy. 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 (from 14 up to 100% of colonies progress). Untransformed clones become senescent (Leboeuf et al., 1990; Pienta et al., 1977; Watanabe & Suzuki, 1991). High frequencies of progression to immortality and anchorage independence were also observed in bulk cultures of SHE cells, where immortality and anchorage independence, a measure of tumourigenicity, were acquired independently by the clones selected from the cultures (4).

9. Evidence indicates that cell transformation results from structural 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 (17). Consistent with these diverse mechanisms, some SHE cell transformants have been shown to harbour biallelic, inactivated p53 tumour suppressor genes (Albor et al., 1994). Carcinogens such as DES can suppress DNA methylation in short-term treatments (Schiffmann et al., 1996). 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 (Isfort et al., 1997). SHE cell transformation by diethanolamine is driven by altered choline metabolism, an important methyl donor in one-carbon metabolism leading to DNA methylation (Lehman-McKeeman & Gamsky, 2000). Introduction of an activated oncogene and a tumour suppressor function, by transfection, will morphologically transform normal SHE cells (Thomassen et al., 1985). Increased frequency of akinetochoric chromosome disjunction occurs during the growth of the initial transformants (Kirchner et al., 1993) which could contribute to the aneuploid characteristic of immortalized clones arising from such populations (reviewed in Ahmadzai et al., 2012).

10. Among later stage immortal and malignantly transformed descendents, global hypomethylation and hypomethylation in ras and myc oncogenes have been observed (Schiffmann et al., 1996; Takahashi et al., 2002). 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 (Yasaei et al., 2013). In morphologically transformed SHE cell lines, cell cycle checkpoint control (G2) is often compromised (Ashra and Rao, 2006). An activated proto-oncogene (cph) capable of transforming other cells has been isolated from malignantly-transformed SHE cells (Notario et al., 1990).

11. 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 (Zhang et al., 2000a, 2000b). 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 (Maire et al., 2005; Alexandre et al., 2003). Growth-factor treatments of SHE cells, presumably acting through signal transduction pathways, can also drive transformation (Isfort 2000) and gap junctional cell-to-cell communication is frequently impaired by non-genotoxic carcinogens (13).

12. Identification of morphologically transformed colonies by microscopic scoring may involve subjectivity, as for any cytohistochemical endpoint. It can be overcome with appropriate training, use of photo catalogues (18) (19) and second opinion or duplicate independent scoring. The assay would be improved by the development of objective measures for scoring transformation, when validated. Some examples include biospectroscopy, which 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).

13. To date comparative sensitivity and specificity of the pH 6.7 and 7.0 versions of the assay (see paragraph 6 above) are limited to a small chemical database (see annex DRP 31(7)). 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.

14. It should be noted that this methods has been validated for substances only and not for mixtures.

PRINCIPLE OF THE TEST METHOD

15. SHE cells are obtained from primary cultures of 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 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.

16. 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 56). 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.

17. 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 substance-treated groups is compared to the frequency of MT colonies in the solvent control group.

PROCEDURE

Culture media, reagents and solutions

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

Culture conditions and counting of viable cells

19. 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

20. SHE cells are isolated from 13-days gestation embryos of pregnant healthy female(s) euthanized 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. Cells can be prepared from single embryos, embryos pooled from a single dam or embryos pooled from different dams sacrificed at the same time.

21. 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 $x10^6$ /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.

22. 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 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

23. 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 foetal bovine serum (FBS) should be checked for suitability. The combination "cell batch/FBS batch" should fulfil the acceptability criteria described in paragraph 56.

Preparation of feeder (irradiated) SHE cells

24. 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. 10 or 40 mL aliquots of cells are transferred to respectively 100 mm culture dishes or T225 culture flasks (2.0 x 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.

25. 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. The temperature is not critical during irradiation. Before and after irradiation, cells should be maintained on ice.

26. 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 (5×10^6 cells/vial) on wet ice, and step frozen (as described in paragraph 22) 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

27. Solid test substances should be dissolved in appropriate solvents and diluted, if appropriate, prior to treatment of the cells. Where this is not possible with compatible solvents, suspensions may need to be used for all the tested concentrations. Liquid test substances may be added directly to the final test medium and/or diluted prior to treatment. Gaseous or volatile substances should be tested by appropriate methods, to be determined on a case by case basis. Fresh preparations of the test substance should be used unless stability data demonstrate the acceptability of storage. A series of solutions at different concentrations of the test substance should be prepared under UV filtered lights or protected from light.

Test conditions

28. The solvent should be chosen to optimize the solubility of the test agent 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, dimethyl sulfoxide. Generally organic solvents should not exceed 0.2% (v/v). This may be achieved by diluting concentrated solutions (500 x) of the test substance 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 34 as examples). If other than well established solvents are used, their use should be supported by data indicating their compatibility with the test substance, the test system, and their lack of transforming potency. In such cases, untreated controls should also be included.

29. The maximum concentrations to be tested in cell transformation assay depend on test substance solubility and cytotoxicity. For test substances 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 substance 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. Paragraph 36 provides further details on the top concentration that should be tested.

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

	Test substance dissolved in		
	Solvent*	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 solvent	100%	0.4%	0.2%

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

Experimental Design

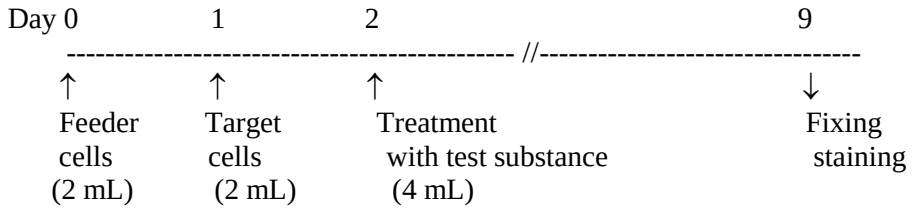

Figure 1: Timeline of the SHE CTA assay (the volumes are per each 60 mm culture dish)

Preparation of test cultures

Feeder layer

30. 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.

31. 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 should form in these dishes.

Target cells

32. 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 56). 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.

33. 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 56). The adjustment of target cell number is explained in paragraph 38.

Treatment of cultures

34. For practicability, one method of preparing dosing solution would be to start with a concentration twice (2 x) the final concentration (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 and dose range finding assay

35. The maximum dose of the test substance should 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 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 38-41).

36. The highest dose level tested for soluble test substances should be 0.01 M, 2 mg/mL, or 2 µL/mL, whichever is the lowest; this applies to chemicals of defined composition. In other circumstances where the test substance is not of 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. In all cases, the test substance solutions are prepared on the day of treatment. The cultures should 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 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.

Selection of treatment concentrations

37. In addition to the controls, at least 5 concentrations to be used in the main experiment are deduced 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 substance does not show a cytotoxic effect, the highest dose is either 0.01 M, 2 mg/mL or 2 µL/mL whichever is the lowest or in certain circumstances at least 5 mg/mL (see Paragraph 36) for a soluble compound, or the visible solubility limit in the final test medium 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

38. 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 56). 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.

39. Twenty four hours after the seeding of the target cells, test and control substance treatment will be started by addition to the test media (4 mL) of the appropriate dosing solution (4 mL) so as to obtain the final concentration (Table 1) in case the procedure proposed as an example is followed (see paragraphs 28 and 34).

40. 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.

41. 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 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

42. All dishes, including those of positive and negative controls, should be coded before microscopic analysis. The stained colonies are 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. (18) and Maire et al. (19), respectively, should be used routinely.

43. 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 dishes should be scored for MT if clearly morphologically transformed.

Generally, for each treatment group ≥ 1000 colonies are evaluated for morphological cell transformation (MT).

44. 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 52-54.

Controls

Solvent control

45. In case the test substance 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%. The final concentration of any solvent should be the same in all solvent control and treated dishes.

Positive control

46. 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 µg/mL in Dimethyl sulfoxide (DMSO) to demonstrate the sensitivity of the assay . However, if justified, other substances can also be considered as positive controls. Each laboratory should establish the performance of the positive control under their own laboratory conditions (see paragraph 51).

Feeder cells control

47. 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

48. The solubility/precipitation of the test substance in the solvent and in the test culture (medium) should be checked and documented.

49. 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 CGM 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. In case of deviation from the expected pH, the pH of the medium should be adjusted to the selected pH. This deviation should be reported and considered in the interpretation of the results.

50. 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.

Proficiency of the laboratory

51. In order to demonstrate proficiency, a laboratory should perform tests with the four positive chemicals acting via different mechanisms, and the negative chemical, included in Table 2. 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.

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 Test Guideline, it is recommended that personnel be trained in a laboratory experienced in this assay.

Table 2: Chemicals for Assessing Laboratory Proficiency

Category	Chemical	CASRN
1. Carcinogens		
	Benzo[a]pyrene	50-32-8
	2,4-diaminotoluene	95-80-7
	3-methyl cholanthrene MCA	56-49-5
	o-toluidine	636-21-5
2. Non carcinogen		
	Anthracene	120-12-7

EVALUATION CRITERIA AND REPORTING

Morphological transformation

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

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

Cytotoxicity

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

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

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

$$RPE = \frac{\text{PE of treatment group}}{\text{PE of the solvent control group}} \times 100$$

55. 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

56. 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. Less than 40 plates are acceptable in this case.

- An average of 25-45 colonies per dish should be available (25). 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 ≥ 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 substance. If the feeder cells are affected by the test substance, 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 of all experienced laboratories and data from the ECVAM validation study, the upper limit of transformation frequency in the negative controls (untreated and solvent) is 0.6%.

- The positive control substance should lead to a biologically relevant and statistically significant increase in the morphological cell transformation compared to the solvent control.

Data interpretation criteria

57. Both statistical and biological relevant data are considered in the interpretation of the negative and positive results, especially the level of concentration(s) increasing the MTF taking into account the range of cytotoxic/non-cytotoxic concentrations. Biological relevance of the results should be considered first.

58. Providing that all acceptability criteria are fulfilled, the following criteria are considered for the evaluation of results:

(1) the increase 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 of the historical negative control data (e.g. 95% confidence interval).

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

60. A test substance is considered as a clear negative if all criteria above (paragraph 58) are not met.

61. 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 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.

62. When results do not meet the criteria for a clear positive or a clear negative call, the test substance should be evaluated by expert judgement and/or 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

63. 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 (if appropriate)
- justification for choice of 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

- identification of serum (provider and batch number)

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 solvent 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
- signs of precipitation
- pH, osmolality of culture media after addition of the test substance
- 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%)

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

1. Barrett J.C. and Ts'o P.O.P. (1978), Evidence for the progressive nature of neoplastic transformation in vitro. Proc. Natl. Acad. Sci., USA, 75: 3761-3765.

2. Kakunaga T. H. Kamasaki H. (1985), Transformation Assay of Established Cell Lines: Mechanisms and Application. IARC Scientific Publications No 67. International Agency for Research on Cancer, Lyon, 225p.

3. Berwald Y., Sachs L. (1963), *In vitro* cell transformation with chemical carcinogens. *Nature*, 200, 1182-1184.

4. Newbold R.F., Overell R.W., Connell J.R. (1982), Induction of immortality is an early event in malignant transformation of mammalian cells by carcinogens. *Nature* 299: 633-635.

5. Elias Z., Poirot O., Pezerat H.,Suquet H., Schneider O ., et al. (1989), Cytotoxic and neoplastic effects of industrial hexavalent chromium pigments in Syrian hamster embryo cells. *Carcinogenesis* 10 (11): 2043-2052.

6. Corvi R., Aardema M.J., Gribaldo L., Hayashi M., Hoffmann S., Schechtman L., Vanparys P., (2012), ECVAM prevalidation study on *in vitro* cell transformation assays: general outline and conclusions of the study. *Mutat. Res.*744, 12–19.

7. OECD (2007), Detailed review paper on Cell transformation assays for detection of chemical carcinogens, OECD Environment, Health and Safety Publications, Series on Testing and Assessment, No. 31 ENV/JM/MONO(2007)18
http://www.oecd.org/officialdocuments/displaydocumentpdf?cote=env/jm/mono(2007)18&doclanguage=en (last accessed 09-09-2011)

8. Vasseur P. and Lasne C. (2012), OECD Detailed Review Paper (DRP) number 31 on "Cell Transformation Assays for Detection of Chemical Carcinogens": main results and conclusions. *Mutat. Res*. 744, 8-11.

9. ECVAM (2011), Recommendation concerning the cell transformation assays using Syrian hamster embryo cells (SHE) and the BALB/c 3T3 mouse fibroblast cell line for in vitro carcinogenicity testing. Annex I: ESAC opinion on the ESAC peer review of an ECVAM-coordinated prevalidation study concerning three protocols of the cell transformation assay (CTA) for in vitro carcinogenicity testing. http://ihcp.jrc.ec.europa.eu/our activities/alt-animal-testing/.

10. Maire M.-A., Pant K., Phrakonkham P., Poth A., Schwind K.-R., Rast C., Bruce S.W., Sly J.E., Bohnenberger S., Kunkelmann T., Schulz M., and Vasseur P., (2012), Recommended protocol for the Syrian hamster embryo (SHE) Cell Transformation Assay, *Mutat. Res.* 744, 76– 81.

11. Pienta R.J., Poiley J.A. and W.B. Lebherz III (1977), Morphological transformation of early passage golden Syrian hamster embryo cells derived from cryopreserved primary cultures as a reliable in vitro bioassay for identifying diverse carcinogens. *Int. J. Cancer*, 19, 642-655

12. Schechtman L.M. (1985), Metabolic activation of procarcinogens by subcellular enzyme fractions in the C3H 10T1/2 and BALB/c 3T3 cell transformation systems. In Transformation Assay of Established Cell Lines: Mechanisms and Application. T. Kakunaga & H. Yamasaki, eds. IARC Scientific Publications No 67, International Agency for Research on Cancer, Lyon, p 137-162

13. Rivedal E., Yamasaki H., and Sanner T. (1994), Inhibition of gap junctional intercellular communication in Syrian hamster embryo cells by TPA, retinoic acid and DDT. *Carcinogenesis*, 15: 689-694.

14. Combes R., Balls M., Curren R., Fischbach M., Fusenig N., Kirkland D., Lasne C., Landolph J., LeBoeuf R., Marquardt H., McCormick J., Müller L., Rivedal E., Sabbioni E., Tanaka N., Vasseur P., Yamasaki H. (1999), Cell Transformation Assays as Predictors of Human Carcinogenicity – The Report and Recommendations of ECVAM Workshop 39, *ATLA* 27, 745-767.

15. LeBoeuf R.A., Kerckaert K.A., Aardema M.J., Isfort R.J. (1999), Use of Syrian hamster embryo and BALB/c 3T3 cell transformation for assessing the carcinogenic potential of chemicals, in The Use of Short and Medium-term Tests for Carcinogens and Data on Genetic Effects in Carcinogenic Hazard Evaluation. D.B. McGregor, J.M. Rice and S. Venitt, eds. IARC Scientific Publications No. 146, International Agency for Research on Cancer, Lyon, 409-425.

16. Adler S., Basketter D., Creton S., Pelkonen O., van Benthem J., et al. (2011), Alternative (non-animal) methods for cosmetics testing: current status and future prospects-2010, *Arch. Toxicol*. 85, 367–485.

17. Baylin S.B., Ohm J.E. (2006), Epigenetic gene silencing in cancer: a mechanism for early oncogenic pathway addition? Nat Rev Cancer 6:107–116.

18. Bohnenberger S., Kunkelmann T., Perschbcher S., Poth A., Pant K., Bruce S.W., Sly J.E., Schwind K.R., (2012), Photo catalogue for the classification of cell colonies in the Syrian hamster embryo (SHE) Cell Transformation Assay at pH 6.7, *Mutat. Res*. 744, 82– 96.

19. Maire M.-A., C. Rast C., P. Vasseur P., (2012), Photo catalogue for the classification of cell colonies in the Syrian hamster embryo (SHE) Cell Transformation Assay at pH 7.0, Mutation Research 744 97– 110.

20. Ahmadzai A.A., Trevisan J., Pang W., Patel II, Fullwood N.J., Bruce S.W., Pant K., Carmichael P.L., Scott A.D., and Martin F.L., 2012. Classification of test agent-specific effects in the Syrian hamster embryo assay (pH 6.7) using infrared spectroscopy with computational analysis. Mutagenesis 27(3):375-382.

21. Landkocz Y., Poupin P., Atienzar F., Vasseur P. (2011), Transcriptomic effects of di-(2-ethylhexyl)-phthalate in Syrian hamster embryo cells: an important role of early cytoskeleton disturbances in carcinogenesis? *BMC Genomics* 12:524.

22. Creton S., Aardema M., Carmichael P.L., Harvey J.S., Martin F.L., Newbold R.F., O'Donovan M.R., Pant K., Poth A., Sakai A., Sasaki K., Scott A.D., Schechtman L.M., Shen R.R., Tanaka N. and Yasaei H, (2012), Cell transformation assays for prediction of carcinogenic potential: state of the science and future research needs, *Mutagenesis*, 27,. 93–101.

23. Vanparys P., Corvi R., Aardema M.J., Gribaldo L., Hayashi M., Hoffmann S. and Schechtman L., (2012), Application of in vitro cell transformation assays in regulatory toxicology for pharmaceuticals, chemicals, food products and cosmetics. *Mutat. Res*. 744, 111-116.

24. OECD (2000), *Guidance document on the recognition, assessment, and use of clinical signs as humane endpoints for experimental animals used in safety evaluation*, OECD Environment, Health and Safety Publications, Series on Testing and Assessment, No. 19 ENV/JM/MONO(2000)7.

25. Kerckaert, G.A., Isfort, R.J, Carr, G.J., Aardema, M.J., LeBoeuf, R.A. (1996), A comprehensive protocol for conducting the Syrian hamster embryo cell transformation assay at pH 6.70 (1996), *Mutat. Res. – Fund. Mol. Mech. Mutagen* 356, 1, 65-84.

26. Jacquet N., Maire M.A., Landkocz Y., P. Vasseur (2012), Carcinogenic potency of perfluorooctane sulfonate (PFOS) on Syrian hamster embryo (SHE) cells. Arch. Toxicol. 86, 2, 305 – 314.

27. Jacquet N., Maire M. A., Rast C., Bonnard M., & P. Vasseur (2012), Perfluorooctanoic acid (PFOA) acts as a tumor promoter on Syrian hamster embryo (SHE) cells. Environ Sci Pollut Res 19, 2537–2549.

28. EURL ECVAM DataBase service on Alternative Methods to Animal Experimentation (DB-ALM) protocol No. 136 on in vitro Syrian embryo hamster cell transformation assay (http://ecvam-dbalm.jrc.ec.europa.eu/).

Ahmadzai, A.A., Trevisan, J., Fullwood, N.J., Carmichael, P.L., Scott, A.D., Martin, F.L. (2012), The Syrian hamster embryo (SHE) assay (pH 6.7): Mechanisms of cell transformation and application of vibrational spectroscopy to objectively score endpoint alterations Mutagenesis, 27 (3), pp. 257-266.

Albor, A., Flessate, D.M., Soussi, T., Notario, V. (1994), 3-Methylcholanthrene inactivates the p53 gene in syrian hamster embryo fibroblasts by inducing a specific intronic point mutation, Cancer Research, 54 (16), pp. 4502-4507.

Alexandre, S., Rast, C., Maire, M.A., Orfila, L., Vasseur, P. (2003), ZnCl2 induces Syrian hamster embryo (SHE) cell transformation, Toxicology Letters, 142 (1-2), pp. 77-87.

Ashra, H., Rao, K.V.K. (2006), Elevated phosphorylation of Chk1 and decreased phosphorylation of Chk2 are associated with abrogation of G2/M checkpoint control during transformation of Syrian hamster embryo (SHE) cells by Malachite green, Cancer Letters, 237 (2), pp. 188-198.

Isfort, R.J. Mechanisms of cell transformation in the Syrian hamster embryo (SHE) cell transformation system (2000), Annals of the New York Academy of Sciences, 919, pp. 86-96.

Isfort, R.J., Cody, D.B., Kerckaert, G.A., Tycko, B., LeBoeuf, R.A., Role of the H19 gene in Syrian hamster embryo cell tumorigenicity, (1997), Molecular Carcinogenesis, 20 (2), pp. 189-193.Lehman-McKeeman & Gamsky, 2000.

Kirchner, S., Stopper, H., Papp, T., Eckert, I., Yoo, H.J., Vig, B.K., Schiffmann, D. Cytogenetic changes in primary, immortalized and malignant mammalian cells (1993), Toxicology Letters, 67 (1-3), pp. 283-295.

LeBoeuf, R.A., Kerckaert, G.A., Aardema, M.J., Gibson, D.P., Multistage neoplastic transformation of Syrian hamster embryo cells cultured at pH 6.70 (1990), Cancer Research, 50 (12), pp. 3722-3729.

LeBoeuf R.A., Kerckaert K.A., Aardema M.J., Isfort R.J. (1999). Use of Syrian hamster embryo and BALB/c 3T3 cell transformation for assessing the carcinogenic potential of chemicals. In The Use of Short and Medium-term Tests for Carcinogens and Data on Genetic Effects in Carcinogenic Hazard ENV/JM/MONO(2007)18 135 Evaluation. D.B. McGregor, J.M. Rice and S. Venitt, eds. IARC Scientific Publications No. 146, Lyon, 409-425.

Maire, M.A., Rast, C., Vasseur, P. Di-(2-ethylhexyl)phthalate (DEHP) increases Bcl-2/Bax ratio and modifies c-myc expression in Syrian hamster embryo (SHE) cells (2005), Toxicology Letters, 158 (3), pp. 237-245.

Notario, V., Castro, R., Flessate, D.M., Doniger, J., DiPaolo, J.A. (1990), Frequent activation of non-ras transforming sequences in neoplastic Syrian hamster cells initiated with chemical carcinogens, Oncogene, 5 (9), pp. 1425-1430.

Pienta, R.J., Poiley, J.A., Lebherz III., W.B. (1977), Morphological transformation of early passage golden Syrian hamster embryo cells derived from cryopreserved primary cultures as a reliable in vitro bioassay for identifying diverse carcinogens, International Journal of Cancer, 19 (5), pp. 642-655.

Schiffmann, D., Dekant, W., Dopp, E., Metzler, M., Henschler, D. (1996), Diethylstilbestrol induces stable, inheritable DNA-hypomethylation in Syrian hamster embro cells throughout multistage neoplastic transformation. In Vitro Toxicol., 9 (2) 167-172.

Takahashi, M., Barrett, J.C., Tsutsui, T. (2002), Transformation by inorganic arsenic compounds of normal Syrian hamster embryo cells into a neoplastic state in which they become anchorage-independent and cause tumors in newborn hamsters, International Journal of Cancer, 99 (5), pp. 629-634.

Thomassen, D.G., Gilmer, T.M., Annab, L.A., Barrett, J.C. (1985), Evidence for multiple steps in neoplastic transformation of normal and preneoplastic Syrian hamster embryo cells following transfection with Harvey murine sarcoma virus oncogene (v-Ha-ras), Cancer Research, 45 (2), pp. 726-732.

Watanabe, M., Suzuki, K. (1991), Expression dynamics of transforming phenotypes in X-irradiated Syrian golden hamster embryo cells, Mutation Research - Fundamental and Molecular Mechanisms of Mutagenesis, 249 (1), pp. 71-80.

Yasaei, H., Gilham, E., Pickles, J.C., Roberts, T.P., O'Donovan, M., Newbold, R.F. (2013), Carcinogen-specific mutational and epigenetic alterations in INK4A, INK4B and p53 tumour-suppressor genes drive induced senescence bypass in normal diploid mammalian cells Oncogene, 32 (2), pp. 171-179.

Zhang H., Kamendulis L.M., Jiang J., Xu Y., Klaunig J.E. (2000a), Acrylonitrile-induced morphological transformation in Syrian hamster embryo cells. Carcinogenesis 21, 727-733.

Zhang H., Wu Y., Kamendulis L.M. and Klaunig J.E. (2000b), Morphological transformation by 8-hydroxy-2’-deoxyguanosine in Syrian hamster embryo (SHE) cells. Toxicol. Sci., 56, 303-31.

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 $NaHCO_3$ 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 (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 pure water

- Cell staining solution (*e.g.* 0.4% to 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 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