

DB-ALM Protocol n° 136 : *In vitro* Syrian Hamster Embryo Cell Transformation Assay (SHE CTA)

Cell transformation, Developmental toxicity, Drug Discovery and Activity Testing

The Syrian Hamster Embryo (SHE) Cell Transformation Assay (CTA) is a short-term *in vitro* assay recommended as an alternative method for testing of the carcinogenic potential of chemicals (both genotoxic and non-genotoxic). The assay is based on the change of the phenotypic features of cell colonies undergoing the first steps of the conversion from normal cells to neoplastic-like cells with oncogenic properties.

Objective & Application

TYPE OF TESTING	: Screening, Replacement (partial)
LEVEL OF ASSESSMENT	: Toxic potential

Context of Use:

This protocol is a combination of **the SHE CTA at pH 6.7** and **the SHE CTA at pH 7.0** protocols, which were evaluated separately during the prevalidation study coordinated by ECVAM from 2005 to 2010 (Pant *et al.*, 2012; Maire *et al.*, 2012a; EURL ECVAM, 2010a and 2010b). It represents the **EURL ECVAM-recommended, standardised, state-of-the-art protocol for performing the SHE CTA** (Maire *et al.*, 2012b), from which a draft OECD test guideline is being developed for a potential replacement and/or reduction method for *in vivo* carcinogenicity testing (EURL ECVAM, 2012). The cell transformation assays are widely used by academia, the chemical, agrochemical, cosmetic, pharmaceutical and tobacco industries, and contract research organisations in the assessment of the carcinogenic potential of chemicals for regulatory and non-regulatory purpose. CTAs can be used as a stand-alone test or in conjunction with others to generate supporting information in mechanistic studies, efficacy evaluation and for hazard identification and risk assessment. A comprehensive list of various areas of CTAs applications is included in the EURL ECVAM recommendation (EURL ECVAM, 2012) and in Vanparys *et al.* (2012). For the regulatory purpose, the carcinogenicity of pharmaceuticals and chemicals is currently assessed in a rodent *in vivo* bioassay. Given the scientific economic and ethical considerations involved, much effort has been made to develop adequate alternatives. CTAs are of particular value as they can detect both genotoxic and non-genotoxic carcinogens and can be used to evaluate certain classes of chemicals for which the traditional *in vitro* genotoxicity tests have low predictive capacity, for example aromatic amines, for which chemical and cosmetic industries use SHE CTA. The vast complexity of the events leading to the *in vivo* carcinogenesis and the low specificity issue of many *in vitro* methods currently in use imply that no single *in vitro* method provides sufficient information for an unequivocal assessment of the carcinogenicity potential of a substance to fully satisfy the regulatory requirements. However it is conceivable that a weight-of-evidence approach including CTA-based information on the transforming potential of chemicals together with other available information from testing and non-testing approaches may be sufficient for decision-making and in some cases allow waiving the use of the rodent bioassay. In other cases, the confirmatory *in vivo* testing may still be required, but CTA results may allow for a more efficient study design.

Applicability Domain:

There are no known apparent limitations related to specific classes of chemicals that could be tested with the SHE CTA. Detailed Review Paper No.31 published by the Organisation for Economic Co-operation and Development (OECD, 2007) refers to pure chemicals and states that CTAs can be applied to organic and inorganic chemicals and that they can be used to identify genotoxic and non-genotoxic rodent carcinogens. However, the following should be taken into consideration:

- Outside of the selection of pure chemicals falling into the classes used in the ECVAM study (Corvi *et al.*, 2012) and included in the OECD DRP (OECD, 2007), it is recommended to include suitable reference chemicals to verify the reliable performance of the CTA.
- The performance of the CTAs using mixtures and formulations has not been considered in the OECD DRP, but it is plausible that the CTAs may be suitable for their testing (Chouroulinkov and Lasne, 1978; Lasne *et al.*, 1990; Bessi *et al.*, 1995; Elias *et al.*, 1996; Cruciani *et al.*, 1999; Breheny *et al.*, 2005).
- Few results are available from the CTAs of nanoparticles but it is plausible that the CTAs may be suitable for their testing (Ponti *et al.*, 2009).

Résumé

SHE primary cells are one of the several well established *in vitro* cell models used to mimic the transformation process *in vivo*. Other systems include immortal mouse fibroblast cell lines BALB/c 3T3 and C3H10T1/2. Upon treatment with carcinogenic chemicals these cells can be induced to undergo a transformation process. They go through distinct morphological changes which can be monitored and quantified as specific endpoints of the transformation.

The SHE CTA has been used in mechanistic studies of *in vitro* transformation since Berwald and Sachs (1963, 1965) confirmed the earlier key observation by Earle (1943) that morphological changes in cell cultures were associated with the *in vivo* oncogenicity of these cells upon inoculation into animals. These authors demonstrated the oncogenicity of SHE cells which presented a transformed phenotype after *in vitro* exposure to genotoxic and non-genotoxic chemical carcinogens. A close correlation was established between the ability of substances from different chemical classes to induce morphological transformation *in vitro* and tumours *in vivo* (DiPaolo *et al.*, 1969, 1971; Barrett *et al.*, 1978a, 1978b, 1979, 1984).

Transformation of SHE cells is a multistage process that results in the conversion of normal cells into fully malignant cells. A minimum of four phenotypic stages appears to be involved in cell transformation (LeBoeuf *et al.*, 1999), which include:

- (a) a block in cellular differentiation visible as morphological transformation
- (b) the acquisition of immortality (unlimited lifespan), an aneuploid karyotype and genetic instability
- (c) the acquisition of tumourigenicity closely associated with the *in vitro* phenotypes of foci formation, anchorage independent growth in semi-solid agar and autocrine factor production
- (d) malignant growth when cells are injected into a suitable host.

Such effects may be caused by changes in the expression of oncogenes and/or tumour suppressor genes (Isfort and LeBoeuf, 1995), however, the complete mechanism underlying these events is not yet fully understood either in the CTAs or human/rodent carcinogenesis. The first signs of transformation typically observed in the assay are linked to changes in cell behaviour and growth and are characterised by an altered cell morphology and disorganised patterns of cell growth.

Experimental Description

Endpoint and Endpoint Measurement:

In the SHE CTA, both cytotoxicity and morphological transformation endpoints are evaluated.

The following parameters are determined for each concentration and control, on the basis of the visual inspection, after scoring of around 1000 colonies per treatment group:

CELLULAR FUNCTIONAL PARAMETERS are scored, which are related to the formation of colonies and transformed loci and include absolute and relative plating efficiency and the frequency of morphological transformation.

COLONY FORMATION - Cytotoxicity assesment is based on the inhibition of colony formation, visible as reduction in the colony number and size.

MORHOLOGICAL EFFECTS - Carcinogenic potential assessment is based on the occurrence of the morphologically transformed colonies.

Endpoint Value:

Plating Efficiency (PE) = $\{[\text{total number of colonies}/\text{total number of target cells seeded}] \times 100\}$

Morphological Transformation Frequency (MTF) = $\{[\text{number of transformed colonies}/\text{total number of colonies}] \times 100\}$ is calculated to measure the occurrence of morphologically-transformed colonies.

Relative Plating Efficiency (RPE) = $\{[\text{PE of treated cells}/\text{PE of control cells}] \times 100\}$ is calculated, in parallel to a qualitative colony density/size evaluation, to measure the in inhibition of colony formation.

Experimental System(s):

SHE cells are primary cells obtained from one or more individual syrian hamsters (*Mesocricetus auratus*) embryos at 13 days of gestation. In most cases one female provides sufficient amount of cells for a conclusive test of one chemical

Bhas 42 cells established from BALB/c 3T3 A31-1-1 cells (BALB/c mouse embryo cells) by transfection of v-Ha-ras oncogene (Sasaki *et al.*, 1990).

Basic Procedure

The CTA is composed of two phases:

- **An initial dose-range finding (DRF)** cytotoxicity test to determine the experimental treatment doses that will be used for the subsequent transformation assay,
- **The transformation assay (TA)**, which represents the main experiment and which includes the measurement of cytotoxicity, the morphological evaluation of individual colonies, and the determination of MTF, in the same dish.

The DRF tests are carried out by the measurement of the PE and the RPE.

The measurement of cytotoxicity during the TA includes RPE and density/size measurements. MTF is calculated as the endpoint for carcinogenic potential.

The overall testing procedure for the DRF and for the TA is similar:

- Briefly, early passage SHE cells are seeded (2 mL volume) on the feeder layer (seeded in 2 mL culture medium) of irradiated SHE cells in (60 mm) dishes, so as to obtain between 25-45 colonies per dish. In TA 40 dishes per dose are seeded to score at least 1000 colonies, per treatment group. In the DRF usually only 10 dishes per dose group are used.
- 24h after seeding, the cells are treated with 4 ml of complete medium containing the test chemical. The cells are exposed to the test chemical for 7 days.
- At the end of the exposure period the medium is removed and the cells are washed with phosphate-buffered saline (PBS), fixed with absolute ethanol and stained with 10% aqueous Giemsa. After rinsing with tap water, the dishes are air-dried before being scored.
- In the DRF, the number of colonies per dish is counted and the reduction in size/density is evaluated. Dose selection for the TA's are based on the results of DRF. In the TA, each dish is coded and scored blindly to eliminate observer bias. The colonies are examined under a stereomicroscope for scoring normal or morphologically transformed phenotypes. The morphologically transformed cells are characterised by a spindle shape, an increased nuclear/cytoplasm ratio and a higher basophilic affinity. These cells have a criss-cross orientation pattern and may be multilayered compared to normal cells.

Controls include:

- A **Positive control**: benzo[a]pyrene (1 or 5 µg/mL dissolved in 0.2% of dimethylsulfoxide (DMSO) for conduct of the assay at pH 7.0 and pH 6.7, respectively).
- A **Vehicle control**: complete culture medium containing the vehicle only, e.g. 0.2% (v/v) of DMSO.
- Additionally, a **feeder cell control** (containing feeder cells but no target cells) is run to check the inability of the feeder cells to replicate.
- An **untreated control** (complete cell culture medium only) can also be run to check the lack of cytotoxicity of the vehicle used.

Around 1000 colonies are scored per concentration for PE, RPE and MTF determinations, in control groups and in each treatment group. A detailed description can be found in the **Procedure details** part of this protocol, under the **Endpoint Measurement** section.

Data Analysis/Prediction Model

The data are analysed using methods established previously (Custer *et al.*, 2000).

First, all results are subjected to the one-sided Fisher's exact test to determine if an increase in morphological transformation occurred compared to the vehicle control (significance level: $p < 0.05$, uncorrected for multiple testing).

The Cochran-Armitage trend test (Armitage, 1955) for a positive dose-related response is to be performed

in cases where only one chemical concentration leads to a statistically significant response (significance level: $p < 0.05$).

Based on the assay results and the statistical analysis, the prediction will be made as follows:

- A test substance will be considered "**negative/non-transforming**" if the percentage of morphologically transformed colonies (MTF) in the test substance treated groups is not statistically significant relative to the concurrent vehicle control (one-sided Fisher's exact test) or it is $\leq 0.6\%$.
- A test substance will be considered "**positive**" if MTF is $> 0.6\%$ and a statistically significant increase in MTF is observed in at least two dose levels compared to the concurrent vehicle control (one-sided Fisher's exact test), **or** if a statistically significant increase in MTF is observed at a single dose level only but with a general positive trend (Cochran-Armitage trend test).
- For results that do not meet the criteria for a clear positive or a clear negative call (**inconclusive results**) the experiment should be repeated.

Test Compounds and Results Summary

Both the SHE CTA at pH 6.7 and the SHE CTA at pH 7.0 variants were evaluated in a multi-laboratory trial with the same six chemicals each (Pant *et al.*, 2012; Maire *et al.*, 2012a; EURL ECVAM, 2010a, 2010b).

The assays were shown to be easily transferable to laboratories that had have basic experience in cell culture techniques but limited experience with such assays. Objectivity and consistency in colony and focus visual scoring is shown to be achievable providing an appropriate training and the use of the photo catalogues produced during the study. Between-laboratory reproducibility is shown to be satisfactory for the two SHE CTA protocol variants.

In addition, the concordance between the different assay results and chemical classification regarding carcinogenicity was examined. Predictive capacity is judged satisfactory:

- 6/6 predictions were correct in the SHE CTA at pH 7.0
- 5/6 predictions were correct in the SHE CTA at pH 6.7

Unexpected results were produced with the non-carcinogen phthalic anhydride, detected as positive in SHE cells at pH 6.7 (Pant *et al.*, 2012; EURL ECVAM, 2010a).

Modifications of the Method

A modification of the SHE CTA method at pH 6.7 exists, which includes a shorter, 24-hour test substance exposure regime (instead of 7 days). In this modification the cells are treated with different concentrations of test substance for 24 hours and at the end of this period, the cells are washed and re-fed with complete culture medium and incubated for six more days. After the six day incubation, the dishes are fixed, stained and scored (Custer *et al.*, 2000). However, this modified method is not the subject of the present protocol as it has not undergone a thorough evaluation and formal validation by EURL ECVAM.

Discussion

Impact on the 3Rs (Replacement, Reduction, Refinement of animal use in experiments)

The use of the SHE CTA has the potential to lead to partial replacement and reduction of animal use (mainly life-time cancer bioassays, OECD, 2009) in the regulatory and non regulatory context (EURL ECVAM, 2012). However, the use of primary cells from Syrian hamster embryos using pregnant female hamsters may be considered sensitive and appropriate methods of humane killing need to be applied.

Practical aspects:

- **Cost:** the CTA is a rather costly *in vitro* test, but cheap in comparison with the rodent bioassay.
- **Throughput:** The CTA requires 2 weeks per substance for combined DRF and TA, which is not very rapid, but still advantageous compared to the 2 years a rodent bioassay requires.
- **Complexity:** the CTA requires high skills with regard to:
 - handling of numerous cell plates simultaneously for several consecutive days,
 - visual scoring

Training and the use of the photo catalogues (Bohnenberger *et al.*, 2012; Maire *et al.*, 2012c) are essential for overcoming these potential limitations.

- **X-ray:** the need for X-ray exposed feeder cells to support the growth of target cells requires the access of an irradiation facility.

Status

Known Laboratory Use:

The cell transformation assays has been widely used worldwide in both academia and industry for over five decades (Schechtman, 2012).

Participation in Evaluation Studies:

OECD Detailed Review Paper :

Extensive review of the existing data on the performances of the main protocol variants of the SHE CTA was made and evaluated in the OECD Detailed Review Paper (DRP 31; OECD, 2007). The DRP concluded that the SHE CTA performances were sufficiently adequate to recommend the development of an OECD Test Guideline based on the protocols used in the ECVAM prevalidation study (see below).

Participation in Validation Studies:

EURL ECVAM prevalidation study:

EURL ECVAM coordinated, from 2005 to 2010, a formal prevalidation study of the SHE CTA by an independent Validation Management Team (VMT). The study goal was to complement the extensive OECD DRP findings by addressing the issues of protocol standardisation, within-laboratory reproducibility, test method transferability, and between-laboratory reproducibility. The results are summarised in Test Compounds and Results Summary section. The VMT concluded that SHE CTA is reproducible and transferable (Vanparys *et al.*, 2011; Corvi *et al.*, 2012).

ESAC Opinion and EURL ECVAM recommendation:

Following the completion of the CTA prevalidation study, ECVAM Scientific Advisory Committee was requested to conduct a scientific peer review of the study results. The ESAC conclusions were generally in line with those of the VMT regarding the satisfactory results of the SHE CTAs (EURL ECVAM, 2011; EURL ECVAM, 2012).

Based on the above-mentioned documents (*i.e.* ESAC opinion, Study Reports) and other relevant documents, mainly the OECD DRP 31 (OECD, 2007) and the ECVAM Workshop Report on CTAs (Combes *et al.*, 1999), the EURL ECVAM issued a recommendation on the SHE pH 6.7 and SHE pH 7.0 CTAs, underlying the potentially significant impact of the CTAs on the 3Rs. The use of CTA data can potentially lead to a partial replacement or reduction of animal use in a weight-of-evidence approach for hazard identification and risk assessment. EURL ECVAM also recommended for a test guideline for the SHE CTA to be developed by OECD.

Regulatory Acceptance:

The SHE CTA is not a regulatory requirement at present. The possible use of the SHE CTA for regulatory purposes as a part of testing strategies is considered in various areas of application:

- The safety and efficacy evaluation of pharmaceuticals (Jacobson-Kram and Jacobs, 2005)
- Guideline on information requirements and chemical safety assessment under REACH legislation for industrial chemicals (ECHA, 2008).
- The Scientific Committee on Consumer Products (SCCP)'s notes of guidance for testing oxidative hair dyes (SCCP, 2006)
- COLIPA's guidance for testing cosmetics (Pfuhler *et al.*, 2010)

OECD follow-up activities:

The current OECD work plan (OECD, 2010) foresees the development of test guideline for the SHE CTA following the submission by France in 2003. Based on the EURL ECVAM's input, the OECD Working Group of National Coordinators of the Test Guidelines Programme (WNT) convened an expert group to recommend the way forward for CTA test guidelines finalisation. The expert group recommended the development of a combined Test Guideline for the SHE CTAs (pH 6.7 and 7.0) which was agreed by the WNT. The draft Test Guideline was released by OECD for public consultation on 05.06.2012.

Health and Safety Issues

General Precautions

Biological material must be considered as potentially dangerous even if appropriate controls are performed. Observe universal precautions in order to protect yourself and your colleagues. Handle the cells in a Level 2-biology safety room.

It is recommended that protective gloves and laboratory coats should be worn when handling hazardous materials.

It is recommended to work safely with respect to the specific class of chemical or hazard (e.g. use and handle potentially carcinogenic test compounds in a fume hood).

Potentially carcinogenic waste is highly hazardous and may have mutagenic or carcinogenic properties and should be given special attention. After each experiment, chemical treated plates are closed by an adhesive film, placed in a plastic bag and appropriately disposed of, in line with local safety regulations.

Abbreviations and Definitions

3Rs: Replacement, Reduction, Refinement of animal use in experiments

AAALAC: Association for Assessment and Accreditation of laboratory Animal Care International

CMF-HBSS: calcium- and magnesium-free Hank's balanced solution

CMF-PBS: calcium- and magnesium-free phosphate buffered saline

COLIPA: the European Cosmetics Association

CRO: Contract Research Organisation

CTA: Cell Transformation Assay

DMEM-L: Dulbecco's Modified Eagle's Medium with LeBoeuf's modification

DMSO: Dimethylsulfoxide

DRF: Dose Range Finding;

DRP: Detailed Review Paper

ECVAM: European Centre for the Validation of Alternative Methods. From 2011 ECVAM is established as the European Union Reference Laboratory for alternatives to animal testing (EURL ECVAM)

ESAC: ECVAM Scientific and Advisory Committee

EURL ECVAM: the European Union Reference Laboratory for alternatives to animal testing

FBS: fetal bovine serum

IACUC: Institutional Animal Care and Use Committee

MTF: Morphological Transformation Frequency

OECD: Organisation for Economic Co-operation and Development

PBS: Phosphate Buffered Saline

PE: plating efficiency

REACH: Registration, Evaluation, Authorisation and Restriction of CHemicals

RPE: relative plating efficiency

SHE: Syrian hamster embryo cell

SPSF: Standard Project Submission Form

SCCP: Scientific Committee on Consumer Products

TA: Transformation Assay

VMT: Validation Management Team

WNT: OECD Working Group of National Coordinators of the Test Guidelines Programme

PROCEDURE DETAILS, 20 June 2012

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- **The user is given the choice between performing the assay at pH 6.7 or at pH 7.0.** The two variants of the assay are similar and their overall performances are considered equivalent (OECD, 2007). The composition of the culture media used in both variants is the same, except for:
 - sodium bicarbonate concentration and consequently the pH
 - the percentage of serum recommended

The use of one or the other pH variant will mainly depend on the previous experience of the laboratory with the assay. For laboratories new to the assay, it is recommended to start using one pH variant so as **to gain sufficient experience in the conduct of the assay**, especially with regard to **scoring at this pH**, and to build a large dataset that can be used as **reference data**, especially with regard to control values.

- Laboratories that are to implement the SHE CTA should receive **proper training from experienced personnel** in order to gain the necessary level of practice to perform the assay and to correctly identify the different types of colonies at the pH of interest. Some differences exist between both pH variants with regard to cell colony morphology and subsequent classification. It is thus recommended to use as a visual aid the corresponding photo catalogues which are each specific to the SHE CTA at pH 6.7 (Bohnenberger *et al.*, 2012) and pH 7.0 (Maire *et al.*, 2012c), respectively. Both photocatalogues are available from the DB-ALM website (<http://ecvam-dbalm.jrc.ec.europa.eu/>). Go to the section related to the protocol No. 136 and select Related information: **Downloads**.

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Materials and Preparations

Cell or Test System

SHE cells are primary cells obtained from individual Syrian hamster embryos at 13 days of gestation. Before use, SHE cells are checked for their cloning efficiency and susceptibility to cell transformation and then stored in liquid nitrogen. One set of the frozen cells is used as feeder cells, the other set as target cells of the treatment and endpoint measurement in CTA.

Equipment

Fixed Equipment

- laminar flow hood (biohazard type and restricted to cell culture assays)
- cell culture incubators (37°C; 10 ± 0.5% CO₂; ≥85% humidity)
- low-speed centrifuge
- water bath (37°C)
- inverse phase microscope

- micropipettors
- computer
- refrigerator (4°C)
- freezers (-20°C and -80°C)
- liquid nitrogen storage facility
- autoclave (for instruments and for bio-hazardous waste materials)
- balance
- pH meter
- osmometer
- cell counting system
- low energy X-ray irradiation equipment

Consumables

The consumables necessary for the conduct of the assay comprise standard cell culture laboratory equipment (e.g. glassware, filtration systems, tips, pipets, cryostorage vials and cell culture plasticware).

Media, Reagents, Sera, others

- **Culture medium:** DMEM-L (Dulbecco's Modified Eagle's Medium with LeBoeuf's modification), which is a modified formulation of low glucose (1 g/L), 110 mg/L of pyruvate, 4 mM of glutamine, and with **or** without phenol red.
- **Fetal Bovine Serum** (FBS, Perbio, Hyclone) is used for the preparation of the complete medium. The **Complete Culture Medium** is prepared with the addition to the culture medium of 15% and 20% (v/v) of FBS for the SHE pH 7.0 and the SHE pH 6.7 CTAs, respectively.

Additional reagents and solutions recommended for the conduct of the assay:

- **Buffered saline:** e.g. calcium- and magnesium-free Hank's balanced solution (CMF-HBSS) **or** calcium- and magnesium-free phosphate buffered saline (CMF-PBS)
- **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
- **Cell staining solution:** e.g. 0.5% (w/v) trypan blue in buffered saline
- **Fixing solution:** methanol **or** ethanol
- **Colony staining solution:** 10% (v/v) Giemsa in deionized or ultra pure water
- **Cryopreservation medium:** e.g. [culture medium + 10% (v/v) FBS + 10% (v/v) DMSO] **or** [culture medium + 20% (v/v) FBS + 7.5% (v/v) DMSO]
- **Dissociation solution** (e.g. dispase 2 U/mL in buffered saline **or** [1.25% (v/v) Enzar-T, 2.5% (v/v) pancreatin + 2% (v/v) of penicillin 10,000 U/mL and streptomycin 10,000 µg/mL solution] in buffered saline)
- **Wash solution:** buffered saline with 1% (v/v) of penicillin 5,000 U/mL and streptomycin 5,000 µg/mL solution
- **Cell isolation medium (CIM)** is constituted with DMEM (pH 7.0 or 6.7 and containing 1.5g/L or 0.75g/L NaHCO₃ respectively) supplemented with 15% FBS and 1% antibiotics (penicillin 5,000 U/mL and streptomycin 5,000 µg/mL)

Preparations

Media and Endpoint Assay Solutions

- **DMEM-L** can be either in ready-to-use solution (Quality Biologicals, USA) or lyophilised (Invitrogen). The ready-to-use solution already contains 0.750 g/L of NaHCO₃ (for pH 6.7) and it can be supplemented with additional 0.750 g/L of NaHCO₃ for pH 7.0.

In the case of lyophilised medium:

1. The powder is reconstituted with ultra pure water and sterilised by membrane filtration (0.2 µm porosity).
2. The pH is then adjusted with NaHCO₃ (Sigma) at a final concentration of approximately 1.5 g/L for pH 7.0 and 0.750 g/L for pH 6.7 (the exact concentrations of NaHCO₃ must

be determined based on the pH measurement after a prior incubation for several hours in a 10% CO₂ atmosphere).

- The pH of the culture medium is checked after incubation at 37°C with 10 ± 0.5% CO₂ in a humidified incubator for at least 24 hours so as to be 6.7 ± 0.05 or 7.0 ± 0.05 and, if necessary, pH is corrected with a 7.5% (w/v) sodium bicarbonate solution, 1N sodium hydroxide or 1N hydrochloric acid.
- The pH-adjusted culture medium should be stored at 4°C and should be used within 4 weeks. The pH should be checked before use according to instructions in point 3.

- FBS** (Perbio, Hyclone) is used for the preparation of the complete medium. FBS may be inactivated at 56°C for 30 minutes prior to being used. According to the quality of serum batch used, it may be useful to heat inactivate FBS. This step allows the suppression of proteins of the complement system and may increase the bioavailability of the test substances in the medium. The complete culture medium is prepared with the addition to the culture medium of 15% and 20% (v/v) of FBS for the SHE pH 7.0 and the SHE pH 6.7 CTAs, respectively.

Test Compounds

- Test substance solutions are prepared fresh on the day of treatment.
- Test substances are dissolved or suspended in an appropriate vehicle and diluted with complete medium. The vehicle should not interact with test substances or affect cell survival and colony formation.
- For non hydrosoluble test substances the solutions are prepared as follows:
 - a series of concentrated solutions (500X) is prepared in DMSO, as the preferred vehicle, in order to achieve a maximal final concentration of DMSO of 0.2% (v/v) in culture medium.
 - Each dosing solution is then prepared from the dilution of the corresponding concentrated solution in medium at a concentration corresponding to 2× the expected final concentration.
 - 4 mL of each dosing solution are transferred into the corresponding culture dish already containing 4 mL of complete medium with feeder and target cells (final volume 8 mL).
 - When the dosing solution is added into the dish, test substance solubility should be controlled in the medium before incubation of the cultures.

Test substance solution	Vehicle	Dosing solution (4 ml test medium)	Final test medium (8 ml)
Concentration of the test substance	500X	2X	1X
Concentration of the vehicle (v/v)	100%	0.4%	0.2%

Positive Control(s)

Benzo[a]pyrene at 1 or 5 µg/mL dissolved in 0.2% of DMSO for conduct of the assay at pH 7.0 and pH 6.7, respectively.

The positive control is not necessary for the dose-range finding test but mandatory for the transformation assay.

Negative Control(s)

- Vehicle control:** complete culture medium containing 0.2% (v/v) of DMSO.
- Feeder cell control** (containing feeder cells but no target cells): to check the inability of the feeder cells to replicate.
- Untreated control** (complete cell culture medium only): can also be run to check the lack of cytotoxicity of the vehicle used.

Method

Test System Procurement

SHE cells are obtained from primary cell cultures of individual Syrian hamster embryos at 13 days of gestation. Before use, SHE cells are checked for their cloning efficiency and susceptibility to cell

transformation see "Checking of the SHE cells/FBS suitability" in the "Routine Culture Procedure" section) and then stored in liquid nitrogen. One set of the frozen cells is used as feeder cells, the other set as target cells.

- **Sacrifice of hamster to obtain embryos**

1. Pregnant Syrian hamster(s) is (are) obtained from a trusted provider and are cared for in accordance with the laws and regulations of governing authorities and following the institutional AAALAC (Association for assessment and Accreditation of laboratory Animal Care International) and IACUC (Institutional Animal Care and Use Committee) or equivalent recommendations.
2. The animal(s) are euthanised at 13 days of gestation using appropriate methods of humane killing, following high ethical standards, and in compliance with laws and regulations in force.
3. The ventral surface is then swabbed with a 10% (w/v) povidone-iodine solution (Betadine®) or 70% (v/v) ethanol.
4. The abdominal skin is retracted and incised, and the peritoneal cavity is opened under sterile conditions.
5. Uterine horns containing embryos are removed and placed into sterile culture dishes (e.g. 100-mm diameter) containing 10 to 20 mL of cold wash solution.

- **Dissociation of embryonic tissue**

1. The embryos are transferred into the sterile 100-mm culture dishes containing 10 to 20 mL of wash solution and are rinsed twice with the wash solution.
2. The embryos are transferred into new culture dishes containing wash solution.
3. The differentiated organs (head, limbs and viscera) are pulled out from each embryo and discarded.
4. The remaining embryonic tissue is cut into thin pieces (of 1 to 3 mm) with sterile sharp curved scissors.
5. The minced tissues are transferred into Erlenmeyer flask(s) (tip: use one Erlenmeyer flask for one or two embryos from one hamster) containing a magnetic stir bar on a stir plate, and rinsed with wash solution at room temperature or at 37°C at slow stirring speed to remove as many blood cells as possible.
6. Tissue is allowed to settle and wash solution is pipetted off and discarded.
7. Dissociation solution (10-20 mL) is added to the flask and tissues are gently stirred with magnetic stir bar on a stir plate for 5 minutes at room temperature or 37°C.
8. Tissue is allowed to settle and dissociation solution is pipetted off and discarded.
9. Dissociation solution is added to the flask and tissues are gently stirred for 10 minutes at room temperature or at 37°C.
10. Tissue is allowed to settle and supernatant (containing dissociation solution and isolated cells) is carefully pipetted off. Supernatant can be filtered through sterile gauze into sterile centrifuge tubes.
11. Fresh dissociation solution is added to the tissue and gently stirred for 10 minutes.
12. The solution and cells are collected as described in point 10.
13. The dissociation has to be repeated 2-4 times with dissociation solution containing dispase or 5-7 times with dissociation solution containing trypsin.
14. Filtered cell suspensions are transferred to centrifuge tubes containing a small volume of FBS to achieve a final concentration of approximately 5-10% (v/v) before centrifugation. Additional FBS may be subsequently added if necessary. The cell suspension is centrifuged at 180-250× g for 10 minutes at 4°C.
15. The supernatant is discarded and the cell pellet is resuspended in Cell isolation medium. The cell suspensions are pooled and sampled for counting of viable cells with cell staining solution.
16. Cells are then seeded at a density of 2×10⁶ viable cells/culture dish (100-mm diameter) or at 0.133×10⁶ cells per cm² area of the 150 or 225-cm² culture flasks.
17. Culture dishes/flasks are incubated for 24 hours at 37°C and 10 ± 0.5% CO₂.

- **Cryopreservation of SHE cells**

1. When cells are 60-80% confluent, culture medium is removed from the culture dishes/flasks and cell layers are rinsed with buffered saline.

2. Cells are detached with detachment solution and culture medium is added into the dishes/flasks to stop the reaction.
3. Cell suspensions are pooled in centrifuge tubes and centrifuged at 180-250×g for 10 minutes at room temperature.
4. The supernatant is then discarded and cell pellet is resuspended with complete culture medium.
5. The viable cells are counted using cell staining solution and centrifuged at 180-250×g for 10 minutes at 4°C.
6. The cell pellet is resuspended in the cold cryopreservation medium (4°C) to obtain a density of e.g. 1×10^6 or 2×10^6 cells/mL for preparing batches of target and feeder cells, respectively.
7. Cell suspension is then aliquoted into storage vials.
8. Cells are frozen step-wise by placing vials on ice at 4°C for 30 minutes, -20°C for 4 hours and finally at -80°C for one night, prior to transfer and storage under liquid nitrogen.

• Preparation of cryopreserved feeder cells

Feeder cells are SHE cells of the same origin as the actual target cells and, after X-ray irradiation, they are no longer able to replicate but are still viable. Feeder cells are used to support colony growth from target cells and can either be prepared fresh or irradiated in advance and stored in cryovials as follows.

1. Several cryopreserved cell vials obtained as described above are thawed rapidly (e.g. in a water bath at 37°C). One or more vials will be needed for each 100mm culture dish.
2. The cells are transferred into centrifuge tubes containing culture medium and centrifuged at 180-250×g for 10 minutes at room temperature.
3. The supernatant is discarded and the cell pellet is resuspended in culture medium.
4. The viable cells are counted with cell staining solution and 2×10^6 viable cells are seeded per 100-mm culture dish **or** at 0.133×10^6 cells per cm² area of the 150 or 225-cm² culture flasks containing respectively 30 or 45 mL of complete culture medium.
5. Culture dishes/flasks are incubated at 37°C and $10 \pm 0.5\%$ CO₂.
6. When cells are 60-80% confluent, the culture medium is removed from the culture dishes/flasks and cell layers are rinsed twice with buffered saline and detached with detachment solution.
7. Culture medium is added into the dishes and cell suspensions are pooled in centrifuge tubes before centrifugation at 180-250×g for 10 minutes at room temperature.
8. The supernatant is then discarded and the cell pellet is resuspended in complete culture medium.
9. Cells are then transferred into a sterile flask containing complete culture medium (e.g. 460 mL of medium in a 175 cm²-flask) and placed on ice before irradiation.
10. The cells are irradiated at 50 grays or 5000 rads using a low energy X-ray machine.
11. After irradiation, the cells are centrifuged at 180-250×g for 10 minutes at 4°C.
12. The supernatant is discarded and the cell pellet is resuspended in complete culture medium.
13. The cell suspension is sampled to count the number of viable cells with cell staining solution and centrifuged at 180-250×g for 10 minutes at 4°C. The pellet is resuspended in the cold cryopreservation medium to a density of 2×10^6 viable cells/mL.
14. Cell suspension is then aliquoted into storage vials (e.g. 3×10^6 cells/vial for batches of irradiated feeder cells).
15. Cells are frozen step-wise by placing vials on ice at 4°C for 30 minutes, at -20°C for 4 hours and finally at -80°C for one night, prior to transfer and storage under liquid nitrogen.

Routine Culture Procedure

Checking of the SHE cells/FBS suitability

Before use, every new target cell batch should be checked for spontaneous transformation rate, plating efficiency (PE; *i.e.* colony forming ability) and morphological transformation with the standard carcinogen benzo[a]pyrene. In the same way, any new batch of FBS should be checked for suitability.

- This checking can be done by testing the new cells with a FBS batch known to be suitable for the

assay or by testing a new FBS batch with cells known to work well.

- Alternatively, when no suitable cell and FBS batches are available, the new target cell batch can be tested simultaneously with different batches of FBS to select the most appropriate combination.

To be considered suitable for the assay, any combination "cell batch/FBS batch" must be checked and the outcome must fulfill the acceptance criteria, as described below:

1. Based on historical data, the target cell number known to produce about 25 to 45 colonies per 60-mm dish is used to seed the new cells to be tested on top of the feeder cells, using culture medium supplemented with different batches of FBS.
2. The dishes are treated with the positive control benzo[a]pyrene (1 or 5 µg/ml for pH 7.0 and pH 6.7 assays, respectively) and the vehicle control DMSO (0.2%, v/v) for 7 days.
3. The colonies are fixed, stained and the total number of colonies per dish and morphological transformation are scored.
4. The combination "FBS/cell batch" must result in:
 - The production of about 25 to 45 colonies per dish.
 - A Morphological Transformation Frequency (MTF) with the vehicle control $\leq 0.6\%$. This threshold is based on published and historical data (Alexandre *et al.*, 2003; Kamendulis *et al.*, 2002; Cruciani *et al.*, 1999; Engelhardt *et al.*, 2004).
 - A significant increase in MTF with the positive control compared to the vehicle control ($p < 0.05$, one-sided Fisher's exact test).

Test Material Exposure Procedures

The Cell Transformation Assay (CTA) includes a preliminary dose-range finding test (DRF) and a subsequent transformation assay (TA).

1. Dose-range finding test

- A range of at least ten concentrations is tested in parallel to the vehicle control in order to achieve a wide range of concentrations, up to the solubility limit or a maximum concentration of 5 mg/mL or 10mM.
- At least ten dishes are seeded per each concentration tested with feeder cell and, subsequently, target cells (see section **2.3 Experimental procedure** for a detailed description).
- At this stage, the number of target cells seeded is the same in all dose groups and is based on the historical data of the number of target cells seeded giving 25-45 colonies per dish with the vehicle control: this number depends on the individual isolate's plating efficiency and is generally comprised between 80-150 cells per dish.
- The conditions of testing (test medium, incubation conditions and time) are the same as those described for the main experiment, *i.e.* for the transformation assay (described in section **2.3 Experimental procedure**).
- Test substance solutions are prepared fresh on the day of treatment, as described in the "**Preparations**" section under "*Test compounds*".
- The maximum dose of the test substance is determined by the solubility and any relevant cytotoxicity information available on the test substance:
 - The highest dose level tested for water soluble test substances is 5 mg/mL or 10 mM, whichever is lower.
 - For water insoluble substances, the highest dose tested will be limited to the lowest precipitating dose in complete medium, except for chemicals that remain insoluble at any concentrations (*e.g.* fibers) and that would require testing at higher insoluble concentrations to reach sufficient toxicity levels.
- The relative cytotoxicity of each treatment group is measured by the reduction in RPE and/or colony density and size of the treated SHE cells compared to the vehicle control group.

2. Transformation Assay

2.1. Dose selection

Based on the results from the Dose-range finding test, definitive doses for the transformation assay should include a minimum 5 concentrations, out of which:

- At least one dose which has no effect on PE,
- At least four concentrations selected up to the highest dose as defined below:
 - A high dose causing an approximate 50% decrease in RPE or relative colony size/density for toxic and water soluble test substances
 - or a high dose of 5 mg/mL or 10 mM, if the test substance is essentially non-toxic;
 - or a high dose limited to the lowest precipitating concentration for insoluble substances.

2.2. Adjusting target cell seeding numbers

An average of about 25-45 scorable colonies per each dish is considered optimum for the assay and should be obtained to consider the assay as valid.

To achieve this, the number of target cells seeded in the transformation assay must be determined from the results of the preliminary cytotoxicity assay. For test substance doses that are toxic or are expected to be toxic, the number of target cells seeded must be increased accordingly.

This adjustment will be performed when RPE is lower than 70%. For instance, for a RPE of 70%, the number of target cells seeded should be multiplied by about 1.5 compared to the one seeded for the vehicle control; for a RPE of 50%, the number of target cells seeded should be multiplied by 2. For details of RPE calculation - see **Endpoint Measurement Section**.

2.3. Experimental procedure

The experimental procedure of the transformation assay is summarised in Figure 1:

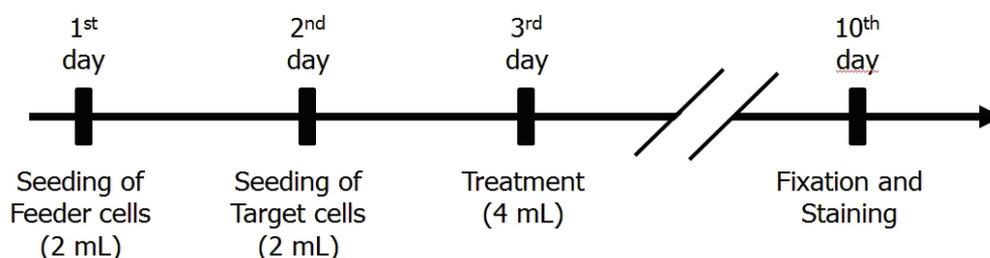


Fig. 1: Experimental procedure of the SHE Cell Transformation Assay. Volumes in brackets correspond to the volume of medium added into the dish at each step.

- On the 1st day of the experiment, 2 mL of SHE feeder cell suspension preliminarily X-irradiated (50 grays or 5000 rad) are seeded in Petri dishes (60-mm diameter).
- The next day (2nd day), 2 mL of target SHE cell suspension are seeded on the feeder layer of irradiated SHE cells (total volume of medium = 4 mL).
- On the 3rd day, i.e. 24h after target cell seeding, the cells are treated with the tested compound by the addition of 4 mL of a 2× solution (total volume of medium = 8 mL).
- The cells are exposed to the chemical for 7 days.
- At the end of the exposure period (10th day) the medium is removed and the cells are washed with phosphate-buffered saline (PBS), fixed with absolute ethanol or methanol, and stained with 10% aqueous Giemsa.

2.3.1. Feeder cell seeding: 1st day

About $4-6 \times 10^4$ feeder cells will be seeded per dish in 2 mL of culture medium and used as a nutrient base for the relatively few target cells and to support the metabolic cooperation. Foresee at least 50 dishes for each treatment group and control.

The feeder cells are prepared fresh 2-4 days before the experiment and are irradiated on the 1st day of the experiment. Alternatively, a stock of irradiated feeder cells may be prepared in advance and stored in cryovials, as described under "**Preparation of cryopreserved feeder cells**" in the "**Test system procurement**" section.

- **If fresh feeder cells are used:**

1. 2-4 days before the 1st day of the assay, a cryovial of SHE cells (2×10^6 cells/vial) from a tested and approved lot from liquid nitrogen storage is thawed and grown to 50-90% confluency in 100-mm culture dishes **or** in 150 or 225-cm² culture flasks containing 10, 30 **or** 45 mL of complete culture medium, respectively.
2. Cells are incubated at 37°C with $10 \pm 0.5\%$ CO₂ in a humidified incubator for 2-4 days.
3. When the cells have reached 50-90% confluency, the medium is removed. The dishes/flasks are washed twice with 10 mL of buffered saline. This is the 1st day of the assay.
4. A single-cell suspension is made by adding 1 **or** 4 mL of detachment solution to the dishes/flasks, respectively, and by subsequent incubation for 2 to 8 minutes at 37°C.
5. To stop the detachment process 1 mL of FBS is added to the dishes/flasks.
6. The dish/flask contents are pooled, mixed gently, transferred into a sterile flask containing complete culture medium (e.g. 10 mL in a 25 cm²-flask) and placed on ice.
7. The cells are irradiated at 50 grays or 5000 rads using a low energy X-ray machine. Between the irradiation periods the flasks are gently shaken and turned in a different direction each time.
8. After irradiation, the cells are transferred into a 50-mL centrifuge tube and centrifuged at 180-250×g for 10 minutes, at 2°C to 8°C.

• **If cryopreserved irradiated feeder cells are used:**

1. Several cryovials (3×10^6 cells/vial) are thawed to obtain enough feeder cells ($4-6 \times 10^4$ per dish).
2. The cells are transferred into a 50-mL centrifuge tube and centrifuged at 180-250×g for 10 minutes at room temperature.

In both cases, after centrifugation:

1. The supernatant is removed and the pellet is resuspended in 30 mL of complete culture medium.
2. The cell concentration and viability are determined by using a hemocytometer and 0.5% cell staining solution.
3. The cell concentration is adjusted to $2-3 \times 10^4$ viable cells/mL in complete culture medium.
4. 2 mL of this suspension (*i.e.* $4-6 \times 10^4$ cells) are added into each 60-mm culture dish.
5. For each assay, at least 40 dishes are prepared for each treatment group and each control (*i.e.* untreated, vehicle and positive controls). In addition at least five dishes per assay are filled with feeder cells only (feeder cell control) to check their inability to replicate: no colony should grow in these dishes.
6. Dishes are incubated at 37°C with $10 \pm 0.5\%$ CO₂ in a humidified atmosphere for 24 hours prior to the addition of target cells.

2.3.2. Target cell seeding: 2nd day

• **preparation 5-24h before the 2nd day of the assay**

1. A cryovial of SHE cells (1×10^6 cells/vial) from a tested and approved lot from liquid nitrogen storage is thawed.
2. Cells are transferred into a centrifuge tube containing 5 mL of complete culture medium and centrifuged at 180-250×g for 10 minutes at room temperature.
3. The supernatant is removed and the cells are resuspended in complete culture medium.
4. Cells are seeded in a 60-mm culture dish, and incubated at 37°C with $10 \pm 0.5\%$ CO₂ in a humidified atmosphere for either five hours or 24 hours.

• **2nd day of the assay**

1. After a pre-incubation period of 5-24 hours, culture medium is removed from the culture dish and cells are rinsed with buffered saline.
2. The target cells are detached with detachment solution and culture medium is added into the dishes to stop the reaction.
3. Cells are counted with a hemocytometer and adjusted with complete growth medium to a concentration sufficient to obtain about 25-45 colonies from 2 mL of the target cell suspension per dish at the end of the test. The target cell number to be seeded is determined on the basis of the results of the DRF test. In case of cytotoxicity expected at certain doses of the test substance, the

target cell number seeded is increased so as to obtain the required number of colonies at the end of the test (see section 2.2).

4. 2 mL of the target cell suspension are added into each culture dishes on the top of feeder cells (total volume 4mL).
5. Dishes are then incubated at 37°C with $10 \pm 0.5\%$ CO₂ in a humidified atmosphere for 24 hours.

2.3.3. Treatment: 3rd day

- Test substance solutions are prepared fresh on the day of treatment, as described in the "Preparations" section under "Test compounds". 4mL of complete medium, containing test substance is added to a test culture dish already containing 4 mL of complete medium with feeder and target cells (final volume 8 mL).
Dishes are then incubated at 37°C with $10 \pm 0.5\%$ CO₂ in a humidified atmosphere for 7 days.
- pH and osmolality:
 - Prior to cell exposure, the pH of the culture medium and of the solution with the test substance needs to be checked after at least 4h of undisturbed incubation at 37°C with $10 \pm 0.5\%$ CO₂ and $\geq 85\%$ humidity in the air to make sure it will remain constant throughout the experiment (pH 6.7 ± 0.05 or pH 7.0 ± 0.05).
 - The pH is checked in parallel with and without the test substance, which should be dissolved in an appropriate vehicle and diluted in complete medium at a concentration equal to the highest concentration to be tested in the cytotoxicity assay.
 - The osmolality is determined using a suitable osmometer. The osmolality is measured prior to or at the end of the exposure time. The dosing solution's osmolality should not be higher than that of the vehicle control by more than 20%.

2.3.4. Fixation and staining: 10th day

1. After the incubation period of 7 days, the medium is aspirated from the dishes and disposed as hazardous waste.
2. The dishes are rinsed with 3-5 mL of buffered saline.
3. After removal of buffered saline, cells are covered with 3-5 mL of fixing solution for at least 10 minutes.
4. Alcohol-containing fixing solution is removed and dishes are air-dried and then stained for at least 20 minutes with 3-5 mL of Giemsa stain (10% in pure water).
5. Stain is then poured away and disposed of as hazardous waste. The dishes are rinsed under tap water and air-dried.

2.3.5. Scoring of the dishes

The stained dishes are blindly scored by examining the colonies under a stereomicroscope for PE and MTF determination.

Endpoint Measurement

In the SHE CTA, both cytotoxicity and morphological transformation endpoints are evaluated.

Both parameters are determined for each concentration and control, on the basis of the scoring of approximately 1000 colonies per group, and after observation under a stereomicroscope and classification of cell colonies as normal or morphologically transformed.

- If a colony contains less than approximately 50 cells, it is not counted.
- Sparse colonies are not scored for MTF evaluation but are included in the total number of colonies for PE calculation.
- Colonies at the edge of the dishes are counted and can be scored for MTF determination if clearly morphologically transformed.

Cytotoxicity

Cytotoxicity assessment is based on the inhibition of colony formation, compared to a vehicle control, as reflected by the PE and the RPE.

- PE = [total number of colonies/total number of target cells seeded] × 100)

- **RPE** = [PE of treated cells/PE of control cells] × 100)
- a qualitative **colony density/size evaluation** is also performed in parallel to characterise colony formation (categorised as normal, slightly reduced *i.e.* 20-30% reduction compared to normal, and greatly reduced *i.e.* 40-60% reduction compared to normal).

Morphological Transformation

Carcinogenic potential assessment is based on the occurrence of morphologically transformed colonies, as reflected by the MTF.

- **MTF** = [number of transformed colonies/total number of colonies] × 100

The classification of colonies is based on the following criteria:

- **Normal phenotype of SHE cell colonies** is characterised by cells which are monolayered, well spread on the dish, organised side by side and properly oriented (*i.e.* there should be a definite orientation of cell flow along longitudinal axis).
- **Morphologically transformed SHE cell colonies** contain cells in an extensive, random-oriented, three-dimensional growth pattern with criss-crossing both at the colony centre and on the perimeter. These cells may be multilayered compared to normal cells (cell stacking). Individual cells within the colony are spindle-shaped, more basophilic relative to their normal counterparts and have a decreased cytoplasm-to-nucleus ratio.

Pictures of both types of colonies can be found in the photo catalogues specific of each variant of the assay (Bohnenberger *et al.*, 2012; Maire *et al.*, 2012a).

Acceptance Criteria

The following criteria must be fulfilled for the validity of the assay:

- **Feeder cells**
 - No colony formation should be observed in the feeder cell control dishes and feeder cells must be visible in the test substance treatment groups except if they are affected selectively by the test substance.
 - If there are colonies in feeder-cell-only dishes, it means that cells have not been sufficiently irradiated. In this case, the assay must be considered invalid.
- **Ability of the target cells to form colonies**
 - For each experiment, PE with the vehicle control should be ≥ 20%.
 - There should be an average of 25-45 colonies per dish for each treatment group. However, in the case of negative results, less than 25 colonies per dish is acceptable, and in the case of a positive result more than 45 colonies per dish are acceptable. It has been shown that an average of less than 25 colonies may increase cell transformation whereas an average more than 45 colonies tends to decrease cell transformation (Kerckaert *et al.*, 1996).
 - The positive control benzo[a]pyrene must lead to a significantly increased MTF compared to that of the vehicle control (one-sided Fisher's exact test, $p < 0.05$).
- **Spontaneous transformation**
 - Based on published data, the acceptable upper limit of spontaneous transformation frequency in the negative controls (untreated and vehicle) is $MTF \leq 0.6\%$.
- **Morphological transformation**
 - The positive control substance must lead to a statistically significant increase of morphological cell transformation frequency ($p < 0.05$, one-sided Fisher's exact test).
 - Each treatment group must contain at least 1000 colonies (less than 1000 colonies are acceptable if the dose group shows a significant increase in the transformation rate;

however the average number of colonies per dish should not be less than 25).

- In case of negative results, there should be at least four scorable (*i.e.* fulfilling the acceptance criteria) concentrations compared to the control.

Data Analysis

Statistical analysis

MTFs obtained for treated cultures and their concurrent experiment controls are compared by means of the **one-sided Fisher's exact test**.

The **Cochran-Armitage trend test** for a positive dose-related response is performed when only one test substance concentration shows a statistically significant response ($p < 0.05$) (Armitage, 1955).

Prediction Model

Based on the assay results and the statistical analysis, the prediction will be made as follows:

- A test substance will be considered "**negative/non-transforming**" if the percentage of morphologically transformed colonies in the test substance treated groups is not statistically significant relative to the concurrent vehicle control (one-sided Fisher's exact test) or it is less than or equal to 0.6%.
- A test substance will be considered "**positive**" if MTF is $> 0.6\%$ and:
 - a statistically significant increase is observed in at least two dose levels compared to the concurrent vehicle control (one-sided Fisher's exact test)
 - or**
 - if a statistically significant increase in MTF is observed at a single dose level only but with a general positive trend (Cochran-Armitage trend test).
- For results that do not meet the criteria for a clear positive or a clear negative call (**inconclusive results**) the experiment should be repeated.

Annexes

Annexes to this protocol can be found on the DB-ALM website (<http://ecvam-dbalm.jrc.ec.europa.eu/>), in the Related information: **Downloads**, under the on-line version of the protocol No. 136: "In vitro Syrian Hamster Embryo Cell Transformation Assay (SHE CTA)", and include:

- The photo catalogues for the SHE pH 6.7 and pH 7.0 CTAs (Bohnenberger *et al.*, 2012; Maire *et al.*, 2012c), recommended with the protocol to support consistency in colony scoring and assay results.
- The reporting templates used during the ECVAM prevalidation study.

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