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Syrian Hamster Embryonic (SHE) cell pH 7.0 Cell Transformation Assay

Prevalidation study Report

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Contents

CONTENTS 2

LIST OF ABBREVIATIONS 5

SUMMARY 6

PREFACE 7

1 RATIONALE FOR THE PROPOSED TEST 8

1.1 INTRODUCTION 8
1.2 INTENDED USE 9
1.3 CURRENT USE 10
1.4 RECENT RESEARCH 10
1.5 OECD DETAILED REVIEW PAPER 11
1.6 PUBLISHED DATA ON BETWEEN-LABORATORY REPRODUCIBILITY 12
1.7 RELEVANT MEETINGS 12
1.8 PATENTS 13

2 ORGANISATION OF THE STUDY 14

2.1 VALIDATION MANAGEMENT TEAM 14
2.2 LABORATORIES INVOLVED 15
2.3 QUALITY SYSTEMS OF THE PARTICIPATING LABORATORIES 15
2.4 CHEMICALS TESTED IN THE SHE CTA PREVALIDATION STUDY 16
2.4.1 Chemical selection 16
2.4.1.1 Selection criteria 16
2.4.1.2 Chemicals selected 16
2.4.2 Modules 2 and 3: Within-laboratory reproducibility and transferability (coded and non-coded chemicals) 17
2.4.3 Module 4: Between-laboratory reproducibility (coded chemicals) 17
2.4.4 Coding/decoding 18
2.5 ASSESSMENT OF WITHIN- AND BETWEEN-LABORATORY REPRODUCIBILITY 18
2.6 STUDY TIMELINE 18

3 MODULE 1: TEST DEFINITION 21

3.1 SCIENTIFIC BASIS FOR THE PROPOSED TEST METHOD 21
3.2 DESCRIPTION OF THE ENDPOINT PREDICTED AND THE MECHANISTIC BASIS OF THE TEST 21
3.3 BIOLOGICAL TEST SYSTEM: SHE CELLS 21
3.4 CELL TRANSFORMATION ASSAY VARIANT: SHE PH 7.0 CTA 22
3.5 PROTOCOL 22
3.5.1 SHE cells 22
3.5.2 Medium 23
3.5.3 Serum 23
3.5.4 Controls 23
3.5.5 Test procedure 23
3.5.6 Statistical analysis of raw data 24
3.5.7 Assay acceptance criteria 24
3.5.8 Assay assessment criteria 25

4 MODULE 2: WITHIN-LABORATORY REPRODUCIBILITY 26

4.1 TRANSFORMATION ASSAY - CODED BENZO(A)PYRENE 26
4.1.1 University of Metz 26
4.1.2 BASF 28
4.1.3 Harlan CCR 28
4.1.4 BioReliance 28
4.1.5 Concurrent cytotoxicity (Relative Plating Efficiency) 29

4.1.6 *Morphological transformation frequency* ... 30
4.1.7 *Acceptance criteria and assessment* ... 31
4.2 CONCLUSION OF THE VALIDATION MANAGEMENT TEAM ON MODULE 2 ... 32

5 MODULE 3: TRANSFERABILITY ... 33

5.1 GENERAL ASPECTS ... 33
5.2 TRAINING ... 33
5.3 CONCLUSION OF THE VALIDATION MANAGEMENT TEAM ON MODULE 3 ... 34

6 MODULE 4: BETWEEN-LABORATORY REPRODUCIBILITY ... 35

6.1 ANTHRACENE ... 35
6.1.1 *Dose-range finding test* ... 35
6.1.2 *Transformation Assay* ... 36
6.1.2.1 University of Metz ... 36
6.1.2.2 BASF ... 36
6.1.2.3 Harlan CCR ... 37
6.1.2.4 BioReliance ... 37
6.1.2.5 Concurrent cytotoxicity (Relative Plating Efficiency) ... 38
6.1.2.6 Morphological transformation frequency ... 38
6.1.2.7 Acceptance criteria and assessment ... 39
6.1.3 *Conclusion* ... 39
6.2 2,4-DIAMINOTOLUENE ... 39
6.2.1 *Dose-range finding test* ... 39
6.2.2 *Transformation assay* ... 40
6.2.2.1 University of Metz ... 40
6.2.2.2 BASF ... 41
6.2.2.3 Harlan CCR ... 41
6.2.2.4 BioReliance ... 42
6.2.2.5 Concurrent cytotoxicity (Relative Plating Efficiency) ... 42
6.2.2.6 Morphological transformation frequency ... 43
6.2.2.7 Acceptance criteria and assessment ... 44
6.2.3 *Conclusion* ... 44
6.3 3-METHYLCHOLANTHRENE ... 44
6.3.1 *Dose-range finding test* ... 44
6.3.2 *Transformation assay* ... 45
6.3.2.1 University of Metz ... 45
6.3.2.2 BASF ... 46
6.3.2.3 Harlan CCR ... 46
6.3.2.4 BioReliance ... 47
6.3.2.5 Concurrent cytotoxicity (Relative Plating Efficiency) ... 47
6.3.2.6 Morphological transformation frequency ... 48
6.3.2.7 Acceptance criteria and assessment ... 49
6.3.3 *Conclusion* ... 49
6.4 O-TOLUIDINE HCL ... 49
6.4.1 *Dose-Range finding test* ... 49
6.4.2 *Transformation assay* ... 50
6.4.2.1 University of Metz ... 50
6.4.2.2 BASF ... 51
6.4.2.3 Harlan CCR ... 51
6.4.2.4 BioReliance ... 52
6.4.2.5 Concurrent cytotoxicity (Relative Plating Efficiency) ... 52
6.4.2.6 Morphological transformation frequency ... 53
6.4.2.7 Acceptance criteria and assessment ... 53
6.4.3 *Conclusion* ... 54
6.5 PHTHALIC ANHYDRIDE ... 54
6.5.1 *Dose-range finding test* ... 54
6.5.2 *Transformation assay* ... 55
6.5.2.1 University of Metz ... 55
6.5.2.2 BASF ... 56
6.5.2.3 Harlan CCR ... 56
6.5.2.4 BioReliance ... 57
6.5.2.5 Concurrent cytotoxicity (Relative Plating Efficiency) ... 57
6.5.2.6 Morphological transformation frequency ... 58

6.5.2.7 Acceptance criteria and assessment 59
6.5.3 Conclusion 59
6.6 OVERVIEW ON VEHICLE AND POSITIVE CONTROLS 60
6.7 CONCLUSION OF THE VALIDATION MANAGEMENT TEAM ON MODULE 4 60
7 SUMMARY OF RESULTS 61
7.1 BENZO(A)PYRENE 61
7.2 ANTHRACENE 61
7.3 2,4-DIAMINOTOLUENE 61
7.4 3-METHYLCHOLANTHRENE 61
7.5 O-TOLUIDINE HCL 62
7.6 PHTHALIC ANHYDRIDE 62
8 OVERALL CONCLUSION BY THE VALIDATION MANAGEMENT TEAM 63
9 RECOMMENDATIONS 64
10 CURRENT CONTACT DETAILS OF THE PEOPLE INVOLVED IN THE PREVALIDATION STUDY 65
11 REFERENCES 66
12 ANNEXES 70
12.1 CHEMICALS SELECTED FOR THE PREVALIDATION OF SHE PH 7.0 CTA 70
12.2 SHE PH 7.0 CELL TRANSFORMATION ASSAY PROTOCOL 71
12.3 REPEATED EXPERIMENT: BASF 2,4-DIAMINOTOLUENE 97

List of Abbreviations

ATR-FTIR	Attenuated Total Reflection Fourier-Transform Infrared
CRO	Contract Research Organisation
CTA	Cell Transformation Assay
DMEM-L	Dulbecco's Modified Eagle Medium with LeBoeuf's modification
DMSO	Dimethyl Sulfoxide
DRF	Dose-Range Finding (test)
DRP	Detailed Review Paper
ECM	Expert Consultation Meeting
ECVAM	European Centre for the Validation of Alternative Methods
ESAC	ECVAM's Scientific Advisory Committee
FBS	Foetal Bovine Serum
FDA	Food and Drug Administration
GLP	Good Laboratory Practice
MTF	Morphological Transformation Frequency
OECD	Organisation for Economic Co-operation and Development
PBS	Phosphate Buffered Saline
PC	Positive Control
PE	Plating Efficiency
REACH	Registration, Evaluation, Authorisation and Restriction of CHemicals
RPE	Relative Plating Efficiency
SCCP	Scientific Committee on Consumer Products
SHE	Syrian Hamster Embryonic cells
SIDS	Screening Information Data Set
SOP	Standard Operating Procedure
TA	Transformation Assay
VC	Vehicle Control
VMT	Validation Management Team

Summary

The potential for a compound to induce carcinogenicity is a crucial consideration when establishing hazard and risk assessment of chemicals and pharmaceuticals, in humans. To date, the standard approach to assess carcinogenicity at a regulatory level is the 2-year bioassay in rodents. The European legislation on chemicals (REACH, Registration, Evaluation, Authorisation and Restriction of Chemicals), cosmetics, pesticides and biocides, all limit the use of animals for safety assessment. In addition, rodent carcinogenicity studies are costly and time consuming and there is a critical need for the availability and implementation of validated alternative test models that can reduce/replace/eliminate the use of animals that would otherwise be employed in carcinogenicity assessments. Several *in vitro* alternatives have been developed for predicting carcinogenicity. Of these, the *in vitro* genotoxicity tests address only one mechanism involved in carcinogenicity, induction of genetic damage. In contrast, *in vitro* cell transformation assays (CTAs) have been shown to involve a multistage process that closely models some stages of *in vivo* carcinogenesis and have the potential to detect both genotoxic and non-genotoxic carcinogens. As such, these tests are currently being used by academia, the chemical, agro-chemical, cosmetic and pharmaceutical industries, and are conducted in-house as well as at CROs to screen for potential carcinogenicity as well as investigate mechanisms of carcinogenicity. CTAs are not used routinely for regulatory testing but they re often used for internal safety assessment of chemicals, drugs, *etc.* and are considered worthwile for providing additional useful information to the prevailing tests that are used for assessing carcinogenic potential.

A recent Detailed Review Paper (DRP) of the OECD (Organisation for Economic Co-operation and Development) on CTAs for the detection of chemical carcinogens (OECD, 2007) concluded that the performance of the Syrian hamster embryo (SHE) and Balb/c 3T3 CTAs were sufficiently adequate and should be developed into formal OECD test guidelines. Further, the same OECD DRP recommended that although considerable and sufficient data on the performance of the assays were available, a formal validation of the assays, in particular focusing on development of a standardised transferable protocol and further information on assay reproducibility, would be important for preparation of such OECD test guidelines. Based on this and previous European Centre for the Validation of Alternative Methods (ECVAM) workshops and expert meetings (Combes *et al.*, 1999), a formal prevalidation study of the CTA using SHE cells at pH 7.0 protocol was conducted following validation modules 1 to 4 of the ECVAM validation procedure (Hartung *et al.*, 2004) in order to evaluate the within-laboratory reproducibility, test method transferability, between-laboratory reproducibility and to develop a standardised state-of-the-art protocol. This prevalidation study is part of a larger program in which two additional variants of the CTA were assessed: the CTA using SHE cells at pH 6.7 and the CTA based on the Balb/c 3T3 A31 cell line.

In keeping with the objectives of this ECVAM's effort, the Validation Management Team (VMT) concluded that the SHE pH 7.0 CTA had been prevalidated in accordance with validation modules 1-4 (Hartung *et al.*, 2004). It has been demonstrated that the assay is reproducible within- and between-laboratories, that it is transferable, and that a standardised protocol is available.

Preface

The study presented in this report complements recent OECD activities related to the cell transformation assays (CTAs). The study has been supervised by a Validation Management Team (VMT) established by the European Centre for the Validation of Alternative Methods (ECVAM). This report includes a short introduction on the context and background of the study, the presentation of the results generated in the prevalidation study and the conclusions and recommendations by the VMT. The conclusions are mainly based on the data generated in this study, but they also take into account the information and experience on the CTA publically available to date. It is the intention of this report to provide data and protocols that further support the consideration of the CTA for use as an alternative method which could contribute to the assessment of the carcinogenic potential of chemicals.

1 Rationale for the proposed test

1.1 Introduction

Development and ultimate utilisation of new chemicals and pharmaceuticals requires, among other prerequisites, the assessment of human safety. One of the main endpoints in this assessment process is the determination of potential carcinogenicity. To date, the standard approach to assess carcinogenicity for regulatory purposes is the 2-year bioassay in rodents (EU Annex V B32, 1998; OECD TG 451, 2008). However, these rodent carcinogenicity assays are associated with technical complexity, high costs and high animal burden, as well as the uncertainty associated to extrapolating from rodent to human. With the entry into force of the new European chemical legislation REACH (Registration, Evaluation, Authorisation and Restriction of Chemicals) (EU, 2006), the 7th Amendment to the Cosmetics Directive (EU, 2003), and the EU revised requirements for pesticides and biocides (EU, 2009), a need for alternatives to routinely employed full animal methods has arisen. The EU Regulation on experimental animals also calls for limiting animal experiments to the extent possible. Among the various *in vitro* alternatives for carcinogenicity prediction developed, the CTAs have been shown to be a multistage process which closely models key stages of *in vivo* carcinogenesis (Landolph, 1985). It is worth mentioning that the CTA is to date the only established and promising *in vitro* assay that has the potential to detect both genotoxic and non-genotoxic carcinogenic compounds. It also appears that the *in vitro* CTA can provide some critical evidence which is specific to the tumourigenic process and that *in vitro* genotoxicity assays cannot provide. Moreover, the test is faster and more cost efficient than the *in vivo* rodent carcinogenicity assay, providing a means for initial screening of chemicals with respect to their carcinogenic potential. As a consequence, data generated using CTAs can facilitate early decision-making as to the need for and/or experimental design of *in vivo* carcinogenicity bioassays.

CTAs are currently being used by academia, the chemical, agro-chemical, cosmetic, pharmaceutical industries, and are conducted in-house as well as at CROs to screen for potential carcinogenicity as well as investigate mechanisms of carcinogenicity. CTAs are considered to provide additional useful information to more routinely employed tests for assessing carcinogenic potential and are therefore listed in various guidelines/testing recommendations for such purposes. Since regulatory agencies receive and review CTA data and these assays are widely used for internal risk assessment of various chemicals, there is a need within the scientific community for standardisation of these test methods and technical guidance on their conduct and use.

This need was already addressed in 1998 by a workshop organised by the ECVAM on CTAs as predictors of human carcinogenicity (Combes *et al.*, 1999). The workshop concluded that the tests indeed are promising but require further development, standardisation and verification. In 2007, the Organisation for Economical Development and Co-operation (OECD) published a detailed review paper (DRP) on the CTAs (OECD, 2007) concluding that the performances of the Syrian hamster embryo (SHE) and Balb/c 3T3 CTAs were sufficiently adequate and these CTA should be developed into formal test guidelines. However, considering the amount of available data reported in the literature, study results have sometimes been generated using different test method protocols. In order to provide a basis for the development of CTA OECD test guidelines, it therefore became important to harmonise and standardise those protocols. Furthermore, as with some other assays with a long history of use, CTAs have not undergone formal validation in accordance with current standards (OECD GD 34, 2005). The previous ECVAM workshop and the recent OECD DRP concluded that a formal validation of the assays, in particular focusing on the use of standardised protocols and reproducibility aspects would be necessary.

With that as a basis and following the recommendations of an expert meeting on cell transformation held at the ECVAM in 2004, ECVAM's next effort was to organise a formal prevalidation study of select CTAs. It was determined that the SHE and Balb/c 3T3 CTAs would undergo a prevalidation

assessment which would address issues of standardisation of protocols, within-laboratory reproducibility, test method transferability, and between-laboratory reproducibility. The results of that study should add to the existing large database of chemicals evaluated over the history of use of these assays (OECD, 2007). In particular, a standard protocol for each of the test methods should be defined which could be used for further development of the sought-after the OECD test guidelines. This exercise started in 2005.

In this prevalidation study three variants of the CTA were assessed: CTA with SHE cells at pH 6.7, CTA with SHE cells at pH 7.0 and CTA using the Balb/c 3T3 cell line. In order to evaluate whether the tests would meet the criteria stipulated by the ECVAM principles on test validity, the modular approach of validation was followed (Hartung *et al.*, 2004). In this study the following modules were assessed:
1) Test definition,
2) Within-laboratory reproducibility,
3) Transferability,
4) Between-laboratory reproducibility.

Due to the specific objectives of this study and the resources available, a limited number of compounds was evaluated as it was not the intention of this study to comprehensively assess the predictive capacity of the CTAs. That would require an exhaustive evaluation of numerous chemicals and chemical classes employing the respective standardised multi-laboratory prevalidated protocols, an effort that was considered beyond the scope of this undertaking. Nevertheless, the data generated by this effort support the assessment of the predictive capacity of the CTAs, a retrospective analysis of which was previously reported by the OECD (OECD, 2007)

Each CTA was conducted following the same agreed upon protocol in at least three different laboratories. The laboratories involved encompassed industry, academia, contract research organisations (CROs) and government establishments located in the USA, Japan and Europe.

The current report, which was prepared by the ECVAM with the support of the VMT, presents the outcome of the prevalidation study of the SHE CTA performed with the pH 7.0 protocol, in four independent laboratories.

1.2 Intended use

The possible use of the SHE CTA is mentioned in various recent testing strategies including the Scientific Committee on Consumer Products (SCCP)'s notes of guidance for testing oxidative hair dyes (SCCP, 2006), as supplemental data for pharmaceuticals (Jacobson-Kram and Jacobs, 2005) and the guidance on information requirements and chemical safety assessment for REACH (ECHA, 2008) and guidance for testing cosmetics (Pfuhler *et al*., 2010). For chemicals produced above 1000 tonnes/year, it is stated that all relevant data from all toxicity studies should be assessed to see whether a sufficiently reliable assessment about the carcinogenicity of the chemical is possible, including alternative means if needed *i.e.* predictive techniques such as chemical grouping and read-across, and the use of (quantitative) structure-activity relationships. On some occasions, it may be proposed to supplement these predictive approaches with short-term tests such as the *in vitro* CTA, cell proliferation assays or medium-term tests like genetically engineered (transgenic) or neonatal models in order to circumvent the need for a chronic carcinogenicity study. This would usually be in the context of adding information to the weight of evidence that a chemical may be carcinogenic.

Based on the performance of the SHE assay, the OECD Expert Consultation Meeting (ECM) in Washington DC, which convened in October 2006 to finalise the OECD DRP on cell transformation (OECD, 2007), recommended that the SHE CTA should be developed into an official OECD Test Guideline. Although there was insufficient information on mechanism of action and usage specific for pharmaceuticals, experts at the Washington ECM were of the opinion that the SHE assay was one

approach (among others) that could be used as a screen in a testing strategy for pharmaceuticals and wasn't therefore limited to non-pharmaceuticals. In addition to its ability to identify potential genotoxic rodent carcinogens, the SHE CTA has shown promise in identifying non-genotoxic carcinogens. It has been proposed for use as a second level *in vitro* screening test for carcinogenic potential or even as a replacement for the *in vitro* mammalian cell genotoxicity assays with similar or lower predictive capacity for chemical carcinogens (OECD, 2007).

1.3 Current use

The SHE CTA is currently being used by academia, chemical, agro-chemical, cosmetic, pharmaceutical and tobacco industries, and CROs to screen chemicals for their potential carcinogenicity. Some current uses of the SHE CTA are: (a) to provide useful ancillary information when the biological significance of the bioassay result is uncertain (*e.g.* in pharmaceutical industry), (b) to clarify *in vitro* genotoxic positive results by weight of evidence (*e.g.* in chemical and cosmetic industries), (c) to evaluate certain classes of chemicals that have a low predictive capacity in the traditional *in vitro* genotoxicity tests (*e.g.* in chemical and cosmetic industries), (d) to screen for non-genotoxic carcinogens (*e.g.* in agro-chemical industry), (e) to demonstrate differences and similarities across a chemical class (*e.g.* in chemical companies within REACH), (f) to screen for efficacy of chemopreventive agents (in pharmaceutical industry), and (g) to investigate tumour promotion activity (*e.g.* in agro-chemical and chemical industries), and (h) for mechanistic studies of carcinogenicity (*e.g.* in academia and industry).

As part of its safety assessment process, submitters have furnished to the FDA (Food and Drug Administration) results from SHE CTA testing as part of the data submission package. Such results are considered as supplemental information in its overall product evaluation (Jacobson-Kram and Jacobs, 2005). However, regulatory agencies in general have been reluctant to unconditionally adopt such assays in their routine safety testing schemes, especially as a full replacement for *in vivo* carcinogenicity testing, due, for the most part, to the lack of formal validation of such assays which demonstrate that the results obtained are equal to or better than that generated *in vivo*. Furthermore, one of the main concerns has been the lack of objective criteria to identify transformed colonies/foci and which could affect the reliability of the test.

1.4 Recent research

This section summarises some of the ongoing and recent research activities related to the SHE CTA (pH 6.7 and pH 7.0).

It is recognised that the visual scoring of the colonies, which is still done manually under the microscope, is one of the greatest weaknesses of this assay. Current developments of the assay include automation of the scoring in order to speed it up and make it more objective and hence more reproducible. Emery *et al.* are currently developing an automated scoring system for SHE pH 6.7 CTA in collaboration with IMSTAR (a high technology company) through a stepwise approach. This includes recognition and capture of the colonies, automated scoring, and recognition of transformed phenotype (presented at the SOT, 2010). In another study, Walsh *et al.* employed attenuated total reflection Fourier-transform infrared (ATR-FTIR) spectroscopy to interrogate pH 6.7 SHE colonies, as complex biomolecules absorb in the mid-infrared giving vibrational spectra associated with structure and function (Walsh *et al*., 2009). Further studies are ongoing in this field. Moreover, it has to be demonstrated whether these approaches are also applicable for the SHE pH 7.0 CTA since colonies display some differences in morphology.

Pant *et al*. have demonstrated the feasibility of conducting the SHE pH 6.7 CTA in cells without using an X-ray irradiated feeder layer, thereby simplifying the test procedure and assisting the scoring of morphological transformed colonies. This eliminates the need for an X-ray machine thereby making

the assay more accessible to laboratories, which is an important consideration if for the assay to be used broadly (Pant *et al*., 2008; Pant *et al*., 2010).

The SHE CTA has been used to study the co-effect of different substances applied simultaneously on cell transformation (Hirose *et al*., 2007). A variant of the SHE CTA using a two-stage protocol has also been applied to cigarette smoke particulates which were found to act both at the initiation and promotion stage of cell transformation (Breheny *et al*., 2005).

Earlier research suggested that SHE cell transformation involves a block in the *in vitro* differentiation of a progenitor cell population (partially differentiated stem cell) present in the SHE cell isolate based on analysis of cellular phenotype, differentiation marker analysis and growth characteristics (Zhang *et al.*, 2004; Nakano *et al*., 1981; Isfort *et al*., 1994; Isfort *et al*., 1996a-b; Kerckaert *et al*., 1996)..

Studies have been conducted to determine the mechanism of induction of SHE cell transformation by specific chemicals. Similar to defining the mechanism of a specific rodent or human carcinogen, case-by-case detailed studies are required..For example, diethanolamine-induced morphological transformation in SHE CTA was shown to be due to a non-genotoxic mechanism involving choline deficiency, consistent with the mechanism of diethanolamine hepatocarcinogenicity in mice (Lehman-McKeeman *et al*., 2000; Lehman-McKeeman *et al*., 2002). Acrylonitrile-induced SHE cell transformation appears to be due to oxidative stress and resulting oxidative damage, and is a mechanism proposed for acrylonitrile-induced carcinogenicity in rats (Zhang *et al*., 2000).

More recent work to define the mechanisms involved in the transformation of the SHE cells have been published and suggest chemical-specific modes of action. Bose *et al*. (2005) indicated that exposure of SHE cells to Malachite green led to elevated phosphorylation of ERK1 and JNK1 and an increase in G2/M phase and apoptotic cells. Maire *et al*. (2005a-b) reported that changes in bcl-2 and bax expression and subsequent dysregulation of apoptosis could contribute to the carcinogenic potential of chemicals such as di(2-ethylhexyl)phthalate and Zinc in SHE cells. Besides, DNA damage and overexpression of the proto-oncogene c-myc, but without any change in apoptosis, were shown in the 2,4-dichlorophenoxyacetic acid-induced SHE cell transformation (Maire *et al*. 2007).

1.5 OECD Detailed Review Paper

Since a number of CTAs have been around for decades and a large number of chemicals have been tested over time using the CTA methods available, the OECD felt it necessary to draft a comprehensive document that captured as much relevant information as possible in order to determine whether the data were sufficient and the time was right to develop appropriate OECD test guidelines for one or more of the CTAs. This DRP, which is an extensive collection of published data evaluating the performance of the different CTAs, provided an overview of the three main types of assays, *i.e.* those which employ (a) the SHE primary cell strain, (b) the Balb/c 3T3 mouse fibroblast cell line and, (c) the C3H/10T½ mouse fibroblast cell line (OECD, 2007). The performance of the SHE pH 7.0 (or higher pH) CTA for the prediction of rodent carcinogenicity was reported for 204 chemicals as follows: concordance 85%, sensitivity 92%, specificity 66%, positive predictivity 88%, negative predictivity 75%, false positive 34%, false negative 8% (the proportion of carcinogens [prevalence] was 74%). Based on the available data the DRP concluded that the performances of the SHE and Balb/c 3T3 CTAs were sufficiently adequate and warranted the development of formal OECD test guidelines. However, to allay any reluctance in drafting such test guidelines and to help ensure that those guidelines were, in fact, developed based upon validated test methods (OECD, 2005), it became apparent that further important information addressing transferability and within- and between-laboratory reproducibility was necessary. Moreover, since in some cases the data evaluated in the DRP had been produced with protocols that had some differences, a goal was to develop standardised and reliable protocols from which the OECD test guidelines would be generated.

In relation to this OECD effort, the development of new test guidelines for SHE and Balb/c 3T3 CTAs have been included in the OECD work plan for the test guidelines programme. These activities will be lead by France and Japan, respectively (OECD, 2009).

1.6 Published data on between-laboratory reproducibility

No formal between-laboratory trial has been previously conducted to fully assess the between-laboratory reproducibility of the SHE CTA at pH 7.0 performed under the same conditions as those evaluated in the present study. However, one review from Isfort *et al.* (1996c) is worth mentioning, which reports a 82% (92/112 chemical or physical agents) inter-laboratory concordance between results obtained using the CTA at pH 7.1-7.3.

The data collected in the OECD DRP for the assessment of the performance of the CTAs enabled an assessment of some measure of reproducibility (OECD, 2007). Excluding chemicals with only one reference, consistency between laboratories for the SHE assay was 87.7% (57/65 chemicals). It should be noted that these results were produced using different variants of the SHE protocol.

1.7 Relevant meetings

ECVAM Workshop, 1998

A workshop on CTAs as Predictors of Human Carcinogenicity held in Angera, Italy, in October 1998 was designed to seek a consensus on the approaches for advancing the use of the *in vitro* mammalian CTAs, with the ultimate goal of (a) achieving regulatory acceptance and implementation of the methodology and (b) reducing the number of animals employed to determine the carcinogenic potential of agents that would otherwise induce malignant tumours in test animals (Combes *et al.*, 1999). By demonstrating a strong correlation between the transformation of mammalian cells *in vitro* and their ability to exhibit neoplasia *in vivo*, one could, hypothetically, rely solely on the *in vitro* endpoint and eliminate animal use and suffering. It is worth noting that the data collected in the OECD DRP were not available at the time of the conduct of this workshop. Among the conclusions and recommendations reached by the workshop, the VMT considered the following as the most relevant ones in relation to this effort:

- Positive rodent CTA data should, in general, be considered to be indicative of a high probability of rodent carcinogenicity, while negative results are indicative of non-carcinogenicity.
- CTAs could provide information which, in combination with data from other testing methods, could be useful for identifying the carcinogenic potential of physical and chemical agents in humans.
- CTAs have the potential to detect various types of carcinogens, including those that are thought to act via genotoxic and non-genotoxic mechanisms.
- A more extensive database on the use of CTAs for screening purposes should be set up, alongside the standard genotoxicity assays (for comparative purposes), by using chemicals with known activities in rodent bioassays. In the longer term, such information should be used to add at least one of the established rodent CTAs (SHE, Balb/c 3T3 or C3H/10T½) to standard carcinogenicity screening packages.
- Consider the need to organise a focused inter-laboratory study involving one or more of the rodent cell-based transformation assays, once they are considered to be ready according to the ECVAM criteria to enter prevalidation.
- The suitability of the currently available rodent protocols for independently-managed inter-laboratory prevalidation studies should be established by ECVAM as a matter of urgency.

ECVAM Expert meeting, 2004

Following the discussions at the OECD and acknowledging the need for alternative methods in the area of carcinogenicity, the ECVAM Task Force on carcinogenicity recommended to bring together a group of experts in the field to discuss whether there was a need to validate the CTA and eventually what should be the involvement of ECVAM. The meeting was held at ECVAM on 15-16 April 2004 and the experts agreed that it was valuable to validate CTAs in accordance with current standards. The funding available at that time for the evaluation of CTAs was only sufficient to conduct the prevalidation of two variants of the assay. For feasibility and practical reasons, the evaluation of the SHE pH 6.7 and the Balb/c 3T3 CTAs was prioritised. In addition, it was agreed that the SHE pH 7.0 protocol would be evaluated by a single laboratory in parallel to the two main studies, due to the amount of valuable SHE pH 7.0 assay historical data available. However, it was clearly stated that the prevalidation of the SHE pH 6.7 and the Balb/c 3T3 CTAs would not exclude that the SHE pH 7.0 CTA and the C3H/10T½ CTA could be subsequently similarly prevalidated, or undergo a catch-up validation, after the first two had undergone scientific prevalidation according to modules 1-4 (Hartung *et al.*, 2004).

1.8 Patents

The test method has not been patented.

2 Organisation of the study

The aim of this prevalidation study was to assess the reproducibility of a SHE CTA using a pH 7.0 standardised protocol. In order to evaluate whether the tests would meet the criteria called for by the ECVAM principles on test validity, the modular approach of validation was followed (Hartung *et al.*, 2004). In this study the following modules were assessed: 1) test definition, 2) within-laboratory reproducibility, 3) transferability, 4) between-laboratory reproducibility. In addition, the data produced are adding to the 5th module on predictive capacity which was in part addressed by the OECD DRP. Each *in vitro* test was conducted according to the same agreed-upon protocol in four different laboratories.

The study was entirely coordinated and sponsored by ECVAM.

This study was organised as described below, taking into account 1) the objective of the study to assess reproducibility of the standardised CTA protocol and not its predictive capacity, which is addressed by the OECD detailed review paper, 2) the high costs and time to perform assays, 3) the limited funding and resources which could be made available by ECVAM and 4) the study design followed for the conduct of the SHE pH 6.7 and Balb/c 3T3 studies. This allowed the evaluation of the CTA using the SHE pH 7.0 protocol in four laboratories, employing six chemicals.

It is important to note that this study should be viewed as one that complements the OECD DRP (OECD 2007) exercise and, in this respect, ECVAM focused on the development of a protocol that could serve as a basis for an OECD test guideline.

2.1 Validation Management Team

Following the principles for test method validation (OECD 34, 2004) an independent VMT was established by ECVAM. Its role was to design the study, to guide and facilitate the prevalidation process, to evaluate the results, and to render subsequent decisions during the progress of the study, and to analyse the outcome. Philippe Vanparys, being member of the ECVAM Carcinogenicity Task Force, was appointed as chairman of the VMT.

Chairman	Philippe Vanparys (J&J PRD, Beerse, Belgium; currently ALTOXICON BVBA, Belgium)
Representative of ICCVAM (until Dec.2006)	Leonard Schechtman (ICCVAM and FDA, USA; currently Innovative Toxicology Consulting, LLC)
Expert	Marilyn Aardema (P&G, USA; currently Marilyn J Aardema Consulting, LLC, USA)
Expert	Makoto Hayashi (NIHS, Japan; currently Biosafety Research Center, Foods, Drugs and Pesticides, Shizuoka, Japan)
Project Management (until April 2008)	Thomas Hartung (ECVAM)
Project Management	Raffaella Corvi (ECVAM)
Project Management & contact person (until March 2007)	Daniela Maurici (ECVAM)
Statistician	Sebastian Hoffmann (ECVAM; currently seh consulting + services, Germany)
Expert	Laura Gribaldo (ECVAM)

The statistical analysis of the *in vitro* data was the responsibility of independent biostatisticians (Sebastian Hoffmann and Andre Kleensang - ECVAM).

B. Claire Thomas (ECVAM from May 2007 to May 2009) and Pascal Phrakonkham (ECVAM since May 2009) assisted ECVAM in the management of the study.

2.2 Laboratories involved

The study included four laboratories from Europe and the USA. The participating laboratories are listed below. Due to its extensive expertise in the assay under validation, Laboratory 1 acted as scientific lead laboratory, while Laboratory 2 had a role of administrative coordinator.

Laboratory 1 (Study Director Paule Vasseur)
(Scientific lead laboratory)
University of Metz
Laboratoire Interactions Ecotoxicité Biodiversité Ecosystèmes (LIEBE) - CNRS UMR 7146
Campus Bridoux, Rue Général Delestraint
Metz 57070, France

Laboratory 2 (Study Directors: Albrecht Poth and Susanne Bohnenberger)
(Administrative coordinator)
Harlan Cytotest Cell Research GmbH
In den Leppsteinswiesen 19
D-64380 Rossdorf,
Germany

Laboratory 3 (Study Director: Kamala Pant)
BioReliance Corporation
14920 Broschart Road
Rockville, MD 20850
USA

Laboratory 4 (Study Directors: Markus Schulz and Karl-Rainer Schwind)
BASF Aktiengesellschaft
GV/TB
67056 Ludwigshafen,
Germany

2.3 Quality systems of the participating laboratories

The present study was conducted under Good Laboratory Practice (GLP)-like conditions by all laboratories according to good scientific practice and good cell culture practice (OECD, 2004). Laboratories 2-4 routinely work under GLP certification and were subjected to regular GLP

inspections while the study was being carried out. Since this was a prevalidation study it was not felt necessary to conduct this study under GLP.

2.4 Chemicals tested in the SHE CTA prevalidation study

The chemicals for the prevalidation study were selected using data from the OECD DRP31 document (draft version August 2004) and the publication by Kirkland *et al.* (2005). Since this prevalidation study was part of a larger project also involving the analysis of the Balb/c 3T3 CTA, the chemical selection took into account existing results in both systems as described below. The same chemicals were used for the prevalidation studies of SHE pH 6.7 CTA and SHE pH 7.0 CTA. Where possible the same chemicals were selected for the evaluation of the SHE and Balb/c 3T3 CTAs.

2.4.1 Chemical selection

2.4.1.1 Selection criteria

The chemicals were selected using the following criteria:

1) Positive both in Balb/c 3T3 and in SHE CTAs,
2) Negative both in Balb/c 3T3 and in SHE CTAs,
3) At least two references available for each test chemical (for both Balb/c 3T3 and SHE),
4) If possible, data available using the SHE pH 6.7 and pH 7.0 protocols,
5) Clear classification as *in vivo* carcinogen or non-carcinogen,
6) Availability of *in vitro* genotoxicity data.

Most of the criteria were met for all chemicals except that only one reference was available for some of the assays: anthracene (only one reference for SHE pH 6.7 and Balb/c 3T3 CTAs), 2,4-diaminotoluene (only one reference for the Balb/c 3T3 CTA), phthalic anhydride (only one reference for both Balb/c 3T3 and SHE pH 6.7 CTAs) and o-toluidine HCl (only one reference for Balb/c 3T3 CTA and no reference for SHE pH 6.7 CTA).

Four of the chemicals selected were in common with those evaluated in the Balb/c 3T3 CTA study, while 2,4-diaminotoluene and phthalic anhydride were used in the SHE studies only, instead of phenanthrene and 2-acetylaminofluorene for which a limited amount of data were available for the SHE CTAs at pH 6.7 and 7.0.

The *in vitro* genotoxicity, *in vivo* genotoxicity and carcinogenicity characterisation of the selected chemicals is reported in Annex 12.1.

2.4.1.2 Chemicals selected

Chemicals selected for the prevalidation study are listed in Table 1.

Table 1: List of chemicals used in the prevalidation study

Chemical	CAS no.	*In vivo* carcinogenicity (References)		Suggested dose range
Benzo(a)pyrene	50-32-8	+	(IARC, 2009)	1 or 5 µg/ml when used as positive control
Benzo(a)pyrene	50-32-8	+	(IARC, 2009)	na when used as coded chemical
Anthracene	120-12-7	-	(IARC, 2009)	na
2,4-Diaminotoluene	95-80-7	+	(IARC, 2009)	na
3-Methylcholanthrene	56-49-5	+	(Gold and Zeiger, 1997)	0.01-10 µg/ml
o-Toluidine HCl	636-21-5	+	(NTP)	20 µg/ml - 1.2 mg/ml
Phthalic anhydride	85-44-9	-	(NTP)	na

na = not applicable

The doses of 3-methylcholanthrene and o-toluidine HCl to be used were suggested by the VMT based on data from the literature to optimise the use of resources (either due to high chemical cost or lack of cytotoxicity) for timely completion of these studies. For the other chemicals the laboratories were asked to select the dose ranges on their own in order to check their ability to identify the critical doses for the transformation assay.

Benzo(a)pyrene was chosen as positive control (PC) because it has been generally reported to induce a strong positive CTA response, in addition to the fact that historical data on this chemical used as PC were available at the lead laboratory.

All chemicals were purchased from Sigma-Aldrich.

2.4.2 Modules 2 and 3: Within-laboratory reproducibility and transferability (coded and non-coded chemicals)

Benzo(a)pyrene was chosen as a coded chemical in this study phase. Benzo(a)pyrene was later used as the PC, in the subsequent phases of the study. Dose ranges for benzo(a)pyrene were suggested by the VMT based on data from the literature.

2.4.3 Module 4: Between-laboratory reproducibility (coded chemicals)

Three chemicals classified as *in vivo* carcinogens (2,4-diaminotoluene, 3-methylcholanthrene, o-toluidine HCl) and two chemicals classified as non carcinogens (anthracene, phthalic anhydride) were selected. Dose ranges for 3-methylcholanthrene and o-toluidine HCl, were suggested by the VMT based on data from the literature. For the other three chemicals, the laboratories had to choose the dose range themselves based on dose-range finding (DRF) tests. The VMT suggested that DMSO (dimethyl sulfoxide) should be used as the vehicle for all chemicals. As the results became available, they were sent to the statistician.

2.4.4 Coding/decoding

All chemicals were coded before sending them to the laboratories. The coding and shipment of chemicals were performed by J&JPRD and ECVAM. The coded chemicals were sent to the laboratory Safety Officers together with the corresponding sealed envelopes containing the Safety Data Sheets. These envelopes were to be opened only in case of accidents and were to be sent back to ECVAM unopened once the experiments were finished. All sealed envelopes were returned to ECVAM at the end of the prevalidation study, except for BioReliance that returned opened envelopes for two chemicals. The safety officer of BioReliance officially certified that the envelopes had been opened by their administration and had not been shared with the laboratories conducting the study. Since the chemicals were coded, the laboratories did not know their identity and therefore all chemicals were treated as potential carcinogens.

The statistical analysis of the data was conducted before the decoding. The chemicals were decoded during the VMT and study directors meeting of January 2009.

2.5 Assessment of within- and between-laboratory reproducibility

Both within- and between-laboratory reproducibility and predictive capacity were evaluated based on concordance of the dichotomous results (negative or positive) as defined by the assessment criteria listed in section 3.5.8.

Regarding the within-laboratory reproducibility, the concordance of results per laboratory was described.

Between-laboratory reproducibility was evaluated by comparing results of the four laboratories.

A preliminary assessment of predictive capacity was described by comparing results with the pre-defined reference results as reported in Table 1, under '*in vivo* carcinogenicity'.

2.6 Study Timeline

The CTA validation studies in SHE cells at pH 6.7 and in Balb/c 3T3 cell line were conducted in the period between 2005 and 2007. In addition, a single laboratory (University of Metz) was selected to evaluate the SHE pH 7.0 protocol in parallel to the other studies.

The results of the above studies were presented and discussed during the VMT and Study Directors meeting and the VMT ECVAM meetings that were held in May 2007 and September 2007, respectively. The results of the CTAs in SHE cells at pH 6.7 and in Balb/c 3T3 cell line SHE cells at pH 6.7 and in Balb/c 3T3 cell line are presented in the accompanying reports. Given the good data produced with the SHE pH 7.0 protocol the VMT recommended a complete assessment of this variant of the test.

A contract for the validation of the SHE pH 7.0 assay between ECVAM and Harlan CCR was signed in December 2007. This study involved the same laboratories that participated to the SHE pH 6.7 study.

As for the SHE pH 6.7 study, an important aspect of the initial phases of the study was the training of the laboratory personnel, including the harmonisation of scoring and of procedures among laboratories. The Standard Operating Procedure (SOP) followed in all laboratories was the one initially used by the Lead Laboratory (University of Metz).

Following the preliminary phase of optimisation of the protocols, the transferability and the within-laboratory reproducibility were assessed by evaluating results obtained for one non-coded test

chemical and a coded one. These two chemicals were the same (benzo(a)pyrene), allowing an analysis of the within-laboratory reproducibility as well as the transferability of the assay. Benzo(a)pyrene was then used as PC in the following phases of the study. After the evaluation of these initial results by the biostatistician and the conclusion by the VMT that the transfer of the tests to the participating laboratories and the within-laboratory reproducibility analysis were successful, the laboratories proceeded to the experimental phase on the between-laboratory reproducibility. The between-laboratory reproducibility was evaluated using five coded chemicals. The chemicals were the same as those used in the SHE pH 6.7 CTA prevalidation study, however this was not known to the participating laboratories and the coding was different.

The data submission template in Excel was developed for each test, in a collaborative effort between the laboratories, ECVAM and the statistician. The spreadsheets containing the test data had to be returned to the statistician of the VMT.

A final signed report for each of the chemicals tested was provided to ECVAM by the Study Directors from the participating laboratories. Moreover, the administrative coordinator laboratory produced a summary report at the end of the study.

At the completion of the study the laboratories were asked to quality check the data that had been analysed by the statistician. They received the sheets with the data used by the statistician and were requested to confirm that the statistician had, in fact, used the right data. The laboratories also quality checked the data presented in this report.

Table 2 summarises the timeline of the study.

Table 2: Timeline of the study

Date	**Location**	
09/2005-05/2007	University of Metz, France	University of Metz assessed the pH 7.0 protocol in parallel to the full prevalidation study carried out on SHE pH 6.7 CTA (for detailed timelines see report on SHE pH 6.7 CTA prevalidation).
09/2007	VMT meeting ECVAM, Ispra, Italy	Agreement to initiate a complete assessment of SHE pH 7.0 protocol, based on good results produced by University of Metz.
18/12/2007	-	Contract signature.
01-03/2008	BioReliance, USA	Isolation of cells.
01-06/2008	All laboratories	Protocol optimisation and start of preliminary experiments.
07/2008	Training University of Metz, France	Training on the conduct of the test method and on colony scoring.
09-12/2008	All laboratories	Performance of main experiments with coded chemicals.
28-29/01/2009	VMT and study Directors meeting ECVAM, Ispra, Italy	Evaluation and discussion of results and decoding of chemicals. One additional test requested by the VMT.
02-04/2009	Laboratories	Repetition of the test at BASF, finalisation of laboratory reports.
03/2010	VMT meeting ECVAM, Ispra, Italy	Finalisation of the prevalidation report to be submitted to the ECVAM's Scientific Advisory Committee (ESAC).

3 Module 1: Test Definition

The following sections provide information about the scientific purpose of the test and the test procedure.

3.1 Scientific basis for the proposed test method

The proposed test method has the potential:
1) to detect genotoxic carcinogens,
2) to detect non-genotoxic carcinogens,
3) to be used for mechanistic studies of multistage carcinogenesis.

3.2 Description of the endpoint predicted and the mechanistic basis of the test

In vitro cell transformation technology employing cultured mammalian cells has been available for over four decades, since the introduction of the methods for transforming normal diploid hamster cells into tumour cells by Berwald and Sachs (1963, 1965). Heidelberger *et al.* (1983) determined that the majority of cell transformation systems fell into three basic categories:

- cell strains (cells with a limited lifespan),
- cell lines (cells with an unlimited lifespan),
- oncogenic viral-chemical interactions involving cells (Fischer rat embryo cells expressing an endogenous retrovirus, mouse embryo cells expressing the AKR leukemia virus, chemical enhancement of a simian adenovirus, SA7 transformation of Syrian hamster or rat embryo cells).

The SHE CTA is based on the conversion of normal to neoplastic-like colonies of cells having oncogenic properties and provides a system to detect genotoxic as well as non-genotoxic carcinogens (Berwald and Sachs, 1963; Berwald and Sachs, 1965; DiPaolo *et al.*, 1971).
Transformation in SHE cells is a process which has shown multistage transformation from a normal cell to a fully malignant cell. A minimum of four phenotypic stages appears to be involved in cell transformation, which include (a) a block in cellular differentiation visualised as morphological transformation in the SHE CTA, (b) the acquisition of immortality expressed by 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, and (d) malignant growth when cells are injected into a suitable host (LeBoeuf *et al.*, 1999). Such effects are caused by changes in the expression of oncogenes and/or tumour suppressor genes (Isfort and LeBoeuf, 1995), however, the complete mechanisms underliying these events are not yet fully understood either in CTAs or human/rodent carcinogenesis. The earliest observation of morphological transformation typically measured in the assay is characterized by changes in the cellular behaviour and cell growth involving alterations in cellular morphology and disorganised patterns of cell growth. Considerable effort has been invested over time in further characterising and describing the use of the SHE CTAs as reviewed in the OECD DRP (2007).

3.3 Biological Test system: SHE cells

SHE cells derived from embryos of Syrian golden hamsters (13-13.5 day gestation) are diploid and genetically stable cells. The cell population comprises a complex mixture of multiple cell types and cells at various stages in the differentiation process, including progenitor stem cells, determined stem cells and fully differentiated cells, and hence provides a broad spectrum of cellular targets for the neoplastic response. They possess a competent metabolic system and have a finite lifespan in culture.

SHE cells show a high proliferation rate, good plating efficiency (PE = 20-40%) and a low spontaneous transformation frequency.

3.4 Cell Transformation Assay Variant: SHE pH 7.0 CTA

The SHE CTA has initially been developed and carried out in medium which exhibited a pH in the range of 7.0-7.35 (Berwald and Sachs, 1963; DiPaolo *et al.*, 1971; Pienta *et al.*, 1977). Thus, a large amount of historical data available have been generated with protocols at pH 7.0-7.35.

The protocol and the criteria for CTA in SHE cells at pH 7.0 are identical to that of the modified version of the protocol where the pH was reduced to 6.7 (Leboeuf and Kerckaert, 1987), except that medium is adjusted to pH 7.0 and the scoring is perceptibly different. At pH 7.0, control colonies are monolayered and they show a high cytoplasm/nucleus ratio and their phenotype is conformed to the phenotype of any fibroblastic or epithelial normal cell culture obtained at physiological pH. Therefore, it is easy for a non-experienced laboratory to discriminate between control and morphologically transformed colonies. It is quite pertinent to carry out experiments at pH 7.0 since this is representative of physiological pH. In case of ionisable chemicals, whose ionisation depends on the pH, it is important to have a culture medium close to physiological pH so as to have a bioavailable fraction which corresponds to the one found at physiological pH.

3.5 Protocol

The detailed protocol is described in Annex 12.2.

The SHE CTA protocol used in this prevalidation study is almost identical to the SHE CTA protocol at pH 6.7, except that the pH of the medium used needs to be adjusted to pH 7.0 with the adequate concentration of sodium bicarbonate.

3.5.1 SHE cells

SHE cells were prepared by BioReliance (USA), following the protocol described in detail in Annex 12.2.

These cells (isolate no.021208) were isolated at pH 7.2-7.3 from the embryos from four female hamsters that were 13-day pregnant on day of arrival (received at BioReliance on 12 February 2008 from Harlan Sprague-Dawley). They were subsequently pooled allowing the preparation of a sufficient number of batches with the same quality and performance. After growth *in vitro* to form sub-confluent monolayer cultures and subsequent harvest they were cryopreserved in liquid nitrogen (13 February 2008). Prior to using this isolate in the transformation assay, the isolate was tested for its transformation properties. Ampoules of cryopreserved cells were shipped in liquid nitrogen to all the laboratories involved in the study.

The same cells were used by all laboratories for both phases of the prevalidation study, except for University of Metz that used target SHE cells isolated at pH 7.0 in its laboratory (batch of cells isolated in February 2006 and selected in May 2006 employing foetal bovine serum (FBS) Hyclone Perbio AQL 25 247).

Each laboratory irradiated their feeder cells and froze them. All laboratories used the same machine for irradiating, except University of Metz which used a different one.

3.5.2 Medium

During the whole study, Harlan CCR, BioReliance and BASF used the same DMEM-L (Dulbecco's Modified Eagle Medium with LeBoeuf's modification) culture medium (not the same batch) from Quality Biologicals, USA while University of Metz used reconstituted medium from lyophilized DMEM without phenol red (Invitrogen). The medium was adjusted to pH 7.0 by adding sodium bicarbonate.

3.5.3 Serum

Harlan CCR, BioReliance and BASF used the same batch of FBS lot no. APB20666, HyClone catalog no. SH30070.03) for the whole prevalidation study. University of Metz used the batch of serum Hyclone Perbio AQL 25 247 for the whole prevalidation study. The serum was heat inactivated at 56ºC for 30 minutes for all the tests performed.

Prior to being used, the batches of FBS were tested with cell isolates that have been shown to work appropriately in previous studies:
- The target cell number producing 25 to 45 colonies per dish dish with a lot of serum already shown to work appropriately in a previous study was seeded on top of the feeder cells using the serum to test.
- The plates were treated with a positive control (PC, 1 µg/ml of benzo(a)pyrene for University of Metz and 5 µg/ml of benzo(a)pyrene for the other laboratories) and a vehicle control (VC, 0.2% DMSO) for seven days, the colonies were fixed, and the total number of colonies per plate and the morphological transformation frequency (MTF) were scored.

The results obtained with the serum lot tested were checked to meet the following criteria:
- Number of colonies obtained with the VC between 25 and 45
- Colonies normal sized
- Statistically significant increase in the number of morphologically transformed colonies with the PC ($p < 0.05$, one-sided Fisher's exact test) and MTF within historical range for PC
- MTF with the VC within the historical range for VC (• 0.6%).

3.5.4 Controls

Positive Control: benzo(a)pyrene (5 µg/ml dissolved in 0.2% DMSO) was used as the PC in all laboratories, except University of Metz which used 1 µg/ml of benzo(a)pyrene.

Untreated Control: The cell culture medium served as the concurrent negative control.

***Vehicle Control*:** The cell culture medium containing 0.2% DMSO served as the concurrent VC.

3.5.5 Test 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 transformation assay (TA),
- The TA, which represents the main experiment and which includes the measurement of cytotoxicity, the morphological evaluation of individual colonies, and the determination of morphological transformation frequency (MTF), in the same dish.

The DRF tests are carried out by measurement of the Plating Efficiency (PE = [total number of colonies/total number of target cells seeded] × 100) and subsequent assessment of the relative plating efficiency (RPE = [PE of treated cells/PE of control cells] × 100).

The measurement of cytotoxicity during the TA includes RPE and density/size measurements. MTF is calculated as follows: MTF = [number of transformed colonies/total number of colonies] × 100.

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

Briefly, early passage SHE cells were seeded on the feeder layer of irradiated SHE cells into 40 dishes (Ø 60 mm) per dose, so as to obtain between 25-45 colonies per dish and to score at least 1000 colonies, per treatment group. Twenty-four hours after seeding, the cells were treated with 4 ml of complete medium containing the test chemical. The cells were exposed to the test chemical for seven days. At the end of the exposure period the medium was removed and the cells were washed with phosphate-buffered saline (PBS), fixed with absolute ethanol and stained with 10% aqueous Giemsa. After rinsing with tap water, the dishes were air dried before being scored. Each dish was coded and scored blindly. The colonies were 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.

Around 1000 colonies were scored per concentration for PE, RPE and MTF determinations, in control groups and in each treatment group.

At least five concentrations were scored, so typically seven to eight concentrations were tested in order to ensure having the adequate number of scorable (*i.e.* fulfilling the assay acceptance criteria listed in section 3.5.7) concentrations. Definitive assay doses should include if possible: a high dose causing at least a 50% decrease in RPE and/or ≥ 50% reduction in relative colony density/size (by visual appearance), and at least one dose which has no effect on PE. If the test chemical was non-toxic, then at least five concentrations were selected up to a maximum of 5 mg/ml or 10 mM (whichever is lower), solubility permitting. For non-toxic and insoluble test chemicals, the highest dose level tested was within 2-times the visible solubility limit in complete medium. For toxic and insoluble test chemicals, the highest dose level tested caused an approximate 50% decrease in RPE or relative colony density, regardless of the number of insoluble dose levels.

3.5.6 Statistical analysis of raw data

The data were analysed using methods established previously as described most recently in Custer *et al*., 2000. Results were analysed using the one-sided Fisher's exact test to determine if an increase in morphological transformation occurred compared to VC (significance level: $p < 0.05$, uncorrected for multiple testing).

The Cochran-Armitage trend test (Armitage, 1955) for a positive dose-related response was performed when only one chemical concentration was statistically significant (significance level: $p < 0.05$).

3.5.7 Assay acceptance criteria

The following assessment criteria were discussed and agreed by the VMT, although it is important to note that some modifications are described in the Recommendations section (section 9) of this report based upon the outcome of the studies conducted:

- The PE of the untreated/vehicle control should be > 20%,

- No colony formation should be observed in the feeder cell control dishes. Feeder cells must be visible in the chemical treatment groups except if they are affected selectively by the chemical,

- • 1000 colonies per treatment group should be available for MTF assessment (less than 1000 colonies is acceptable if the dose group shows a statistically significant increase in the transformation rate),

- There should be 25-45 colonies per dish for each treatment group. However, in the case of negative results < 25 colonies is acceptable and in the case of a positive result > 45 colonies per dish are acceptable,

- Transformation frequency in the negative controls (untreated and vehicle) must be within the range of historical controls. 0.6% has been chosen as upper limit. This value was based on published data and was consistent with the historical data of the laboratories,

- The PC chemical must lead to a statistically significant increase of morphological cell transformation,

- There should be at least five scorable concentrations.

3.5.8 Assay assessment criteria

The following assessment criteria were agreed by the VMT based on laboratories experience and the literature. These criteria were the same as those used in the SHE CTA pH 6.7 study (most recently Custer *et al*., 2000):

- A test chemical will be considered "negative/non-transforming" if the following criterion is met:
  - the percentage of morphologically transformed colonies in the test chemical-treated groups is not significantly different from that of the concurrent VC or it is less than or equal to 0.6%.

- A test chemical will be considered "positive" if the following criteria are met:
  - a statistically significant increase in transformation frequency (above 0.6% MTF) at at least two dose levels compared to the concurrent VC, or
  - a statistically significant increased frequency in morphologically transformed colonies (above 0.6% MTF) only at a single dose level but with a general positive trend.

4 Module 2: Within-laboratory reproducibility

In order to evaluate the initial within-laboratory reproducibility, the PC chemical, benzo(a)pyrene, was tested in each laboratory as a coded chemical. This section of the report provides these results, including the data of the DRF tests and the TAs of all laboratories.

In addition, benzo(a)pyrene was used as the PC for the assay conducted with coded chemicals and the within-laboratory reproducibility of benzo(a)pyrene was further evaluated (Figure 18).

Figure 1-Figure 2 and Table 3-Table 6 show the results of the TAs. Sparse colonies were not scored for MTF assessment, but were included in the total number of colonies for the calculation of the PE. Therefore the column "scorable colonies" is the total number of colonies minus the sparse colonies. This number is used in the Fisher's exact test to calculate the significance of the increase in MTF.

The cytotoxicity of benzo(a)pyrene is shown by the RPE (%) and colony density/size (normal, slightly or greatly reduced) columns.

The other columns indicate the number of morphologically transformed colonies, the percentage of morphologically transformed colonies compared to the total number of scorable colonies and the results (*p*-value) of the Fisher's exact test.

4.1 Transformation Assay - coded benzo(a)pyrene

Benzo(a)pyrene was assessed for its potential to induce morphological transformation in early passage SHE cells after an exposure time of seven days. Each laboratory carried out initial DRF cytotoxicity tests to determine the experimental doses. The VC (DMSO) gave transformation frequencies within the expected range for the SHE cells under the assay conditions employed (• 0.6%): 0.26-0.45% (University of Metz), 0.08% (BASF), 0.34% (Harlan CCR) and 0.42% (BioReliance). The PC chemical (benzo(a)pyrene) led to the expected increase in morphologically transformed colonies. Further evidence of within-laboratory reproducibility is seen in the results for benzo(a)pyrene used as the PC in the studies described in Module 4 below.

4.1.1 University of Metz

The within-laboratory reproducibility of the SHE pH 7.0 CTA protocol was initially assessed by University of Metz only (2005-2006), in parallel to the evaluation of the SHE pH 6.7 CTA protocol. Since at that time University of Metz was the only laboratory to assess the pH 7.0 protocol, the VMT requested that the laboratory should perform three independent assays using coded benzo(a)pyrene. Upon the demonstration that the assays performed with the pH 7.0 protocol were reproducible, the laboratory could proceed to the next phase of the prevalidation.

In the preliminary phase of optimisation, University of Metz used the cells that had been isolated by BioReliance, grown and stored at pH 6.7. These cells showed not to be appropriate for the conduct of the assay at pH 7.0 indicating the importance of isolating the cells at the pH that will be used for the conduct of the assay. Therefore, University of Metz subsequently used cells isolated by their own laboratory at pH 7.0 and modified the SOP (Standard Operating Procedure) to include the protocol of cell isolation and preparation.

In the following table (Table 3) the measurements of RPE and MTF of the experiments are summarised. Based upon the results of the preliminary DRF test, the doses selected for the evaluation of the induction of morphological transformation ranged from 0.01 to 25 µg/ml. The MTF values in the three independent experiments (TA1, TA2 and TA3) ranged from 0.39% to 6.41%, 0.34% to 6.11%,

and 0.45% to 5.13%, respectively. All experiments reproducibly showed significant increases in MTF compared to the VC ($p < 0.01$) at all test chemical concentrations equal to or higher than 0.1 µg/ml. Results of TA1 for 1 µg/ml were not taken into consideration for the final assessment since the average number of colonies per dish was slightly below the limit for a positive result (24.8).

Table 3: Transformation assay results from University of Metz, testing coded benzo(a)pyrene

Uni Metz Coded benzo(a)pyrene (µg/ml) TA1	Scorable colonies	RPE (%)	Colony density/size	Transformed colonies	MTF (%)	Fisher's exact test *p*-value (one-sided)
VC (DMSO 0.2%)	1576	100.0	+	6	0.38	-
0.01	1281	81.0	+	5	0.39	>0.5
0.1	1043	66.1	+	33	3.16	<0.01**
1	993	62.5	+	26	2.62	<0.01**
5	1018	68.7	+	43	4.22	<0.01**
10	1359	87.1	+	35	2.58	<0.01**
15	1297	83.5	+	73	5.63	<0.01**
25	1213	80.6	+	77	6.41	<0.01**

Uni Metz Coded benzo(a)pyrene (µg/ml) TA2	Scorable colonies	RPE (%)	Colony density/size	Transformed colonies	MTF (%)	Fisher's exact test *p*-value (one-sided)
VC (DMSO 0.2%)	1559	100.0	+	4	0.26	-
0.01	1476	94.7	+	5	0.34	>0.5
0.1	1103	71.1	+	22	1.99	<0.01**
1	1100	70.7	+	41	3.73	<0.01**
5	1129	73.3	+	69	6.11	<0.01**
10	1241	79.9	+	45	3.63	<0.01**
15	1196	77.2	+	36	3.01	<0.01**
25	1178	75.8	+	47	3.99	<0.01**

Uni Metz Coded benzo(a)pyrene (µg/ml) TA3	Scorable colonies	RPE (%)	Colony density/size	Transformed colonies	MTF (%)	Fisher's exact test *p*-value (one-sided)
VC (DMSO 0.2%)	1543	100.0	+	7	0.45	-
0.01	1556	100.8	+	7	0.45	>0.5
0.1	1195	77.4	+	21	1.76	<0.05**
1	1210	78.6	+	35	2.89	<0.01**
5	1174	76.7	+	52	4.43	<0.01**
10	1224	80.1	+	61	4.98	<0.01**
15	1190	78.0	+	61	5.13	<0.01**
25	1190	85.2	+	58	4.43	<0.01**

VC = Vehicle Control, RPE = Relative Plating Efficiency, MTF = Morphological Transformation Frequency, + = normal density/size.
** = $p < 0.01$ (one-sided Fisher's exact test)

4.1.2 BASF

The doses selected ranged from 1.25 to 10 µg/ml and the MTF ranged from 0.88% to 1.87% (Table 4). All test chemical concentrations induced significant increases in MTF compared to the VC ($p < 0.01$).

Table 4: Transformation assay results from BASF, testing coded benzo(a)pyrene

BASF Coded benzo(a)pyrene (µg/ml)	Scorable colonies	RPE (%)	Colony density/size	Transformed colonies	MTF (%)	Fisher's exact test *p*-value (one-sided)
VC (DMSO 0.2%)	1277	100.0	+	1	0.08	-
1.25	1018	78.7	+	19	1.87	<0.0005**
2.5	1018	81.1	+	9	0.88	0.004**
5	1016	79.0	+	16	1.57	<0.0005**
7.5	1024	80.6	+	18	1.76	<0.0005**
10	1139	87.2	+	16	1.40	<0.0005**

VC = Vehicle Control, RPE = Relative Plating Efficiency, MTF = Morphological Transformation Frequency, + = normal density/size.
** = $p < 0.01$ (one-sided Fisher's exact test)

4.1.3 Harlan CCR

The test doses ranged from 1.25 to 10 µg/ml and the MTF ranged from 1.45% to 3.29% (Table 5). All test chemical concentrations induced significant increases in MTF compared to the VC ($p < 0.01$), with a dose-dependent effect.

Table 5: Transformation assay results from Harlan CCR, testing coded benzo(a)pyrene

Harlan CCR Coded benzo(a)pyrene (µg/ml)	Scorable colonies	RPE (%)	Colony density/size	Transformed colonies	MTF (%)	Fisher's exact test *p*-value (one-sided)
VC (DMSO 0.2%)	2337	100.0	+	8	0.34	-
1.25	2424	85.7	+	29	1.45	<0.0005**
2.5	2002	91.5	+	56	2.62	<0.0005**
5.0	2138	81.1	+	57	3.01	<0.0005**
7.5	1896	84.1	+	58	2.96	<0.0005**
10.0	1960	91.0	+	70	3.29	<0.0005**

VC = Vehicle Control, RPE = Relative Plating Efficiency, MTF = Morphological Transformation Frequency, + = normal density/size.
** = $p < 0.01$ (one-sided Fisher's exact test)

4.1.4 BioReliance

The test doses ranged from 1 to 10 µg/ml and the MTF ranged from 1.38% to 2.17% (Table 6). All test chemical concentrations induced significant increases in MTF compared to the VC ($p < 0.01$).

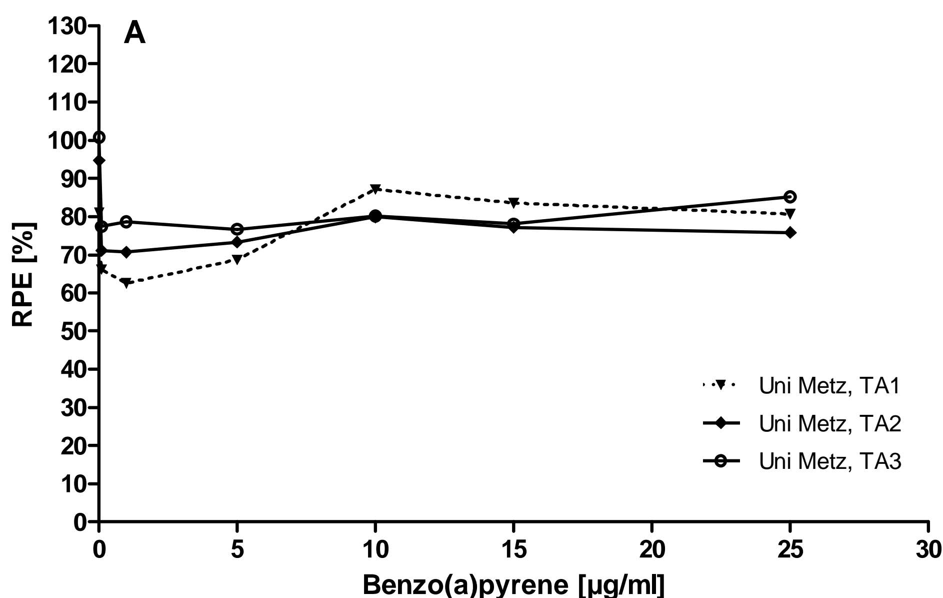
Table 6: Transformation assay results from BioReliance, testing coded benzo(a)pyrene

BioReliance Coded benzo(a)pyrene (µg/ml)	Scorable colonies	RPE (%)	Colony density/size	Transformed colonies	MTF (%)	Fisher's exact test *p*-value (one-sided)
VC (DMSO 0.2%)	1203	100.0	+	5	0.42	-
1.0	1372	114.2	+	19	1.38	0.008**
2.5	1406	117.4	+	20	1.42	0.006**
5.0	1522	126.8	+	33	2.17	<0.0005**
7.5	1472	122.4	+	27	1.83	<0.0005**
10.0	1487	124.1	+	24	1.61	0.002**

VC = Vehicle Control, RPE = Relative Plating Efficiency, MTF = Morphological Transformation Frequency, + = normal density/size.
** = $p < 0.01$ (one-sided Fisher's exact test)

4.1.5 Concurrent cytotoxicity (Relative Plating Efficiency)

Cytotoxicity of benzo(a)pyrene was evaluated by RPE assessment in all laboratories. University of Metz repeated the experiment three times (TA1, TA2 and TA3), whereas BASF, Harlan CCR and BioReliance performed a single experiment (Figure 1).



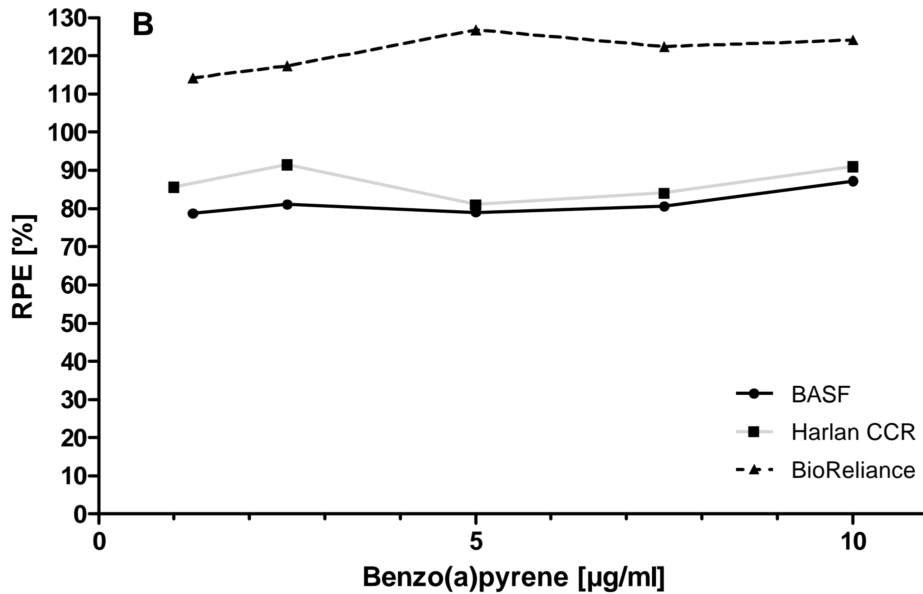
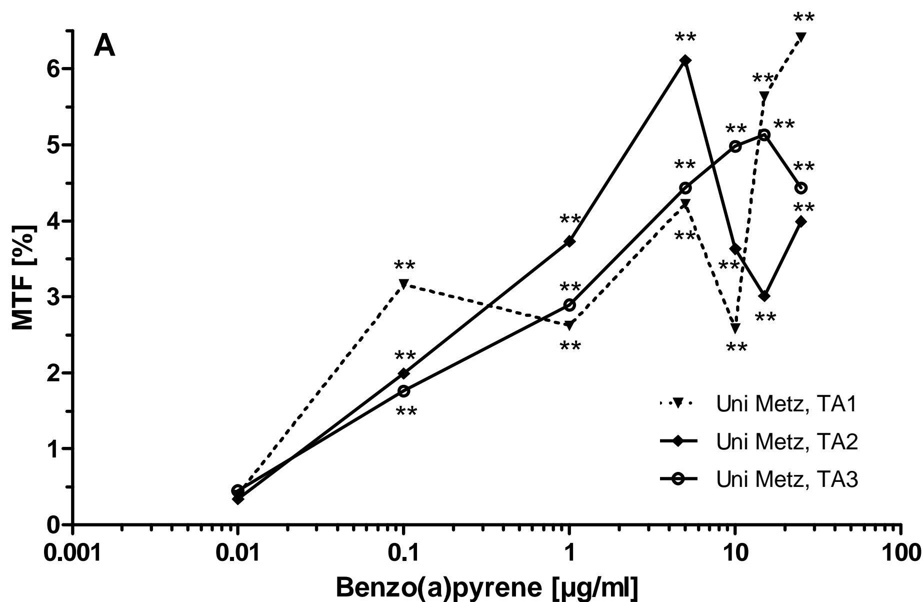


Figure 1: Relative Plating Efficiency (RPE) compared to vehicle control in all laboratories testing coded benzo(a)pyrene: University of Metz repeated the experiment three times (**A**), whereas BASF, Harlan CCR and BioReliance performed a single experiment (**B**)

4.1.6 Morphological transformation frequency

MTF results with benzo(a)pyrene are shown in Figure 2. Benzo(a)pyrene induced a statistically significant increase in morphological transformation compared to VC in all laboratories and at every concentration tested. The shapes of the curves of University of Metz repeated experiments show a clearer dose-dependent increase of MTF in comparison to the curves of the other laboratories, which is in part due to testing at lower doses.



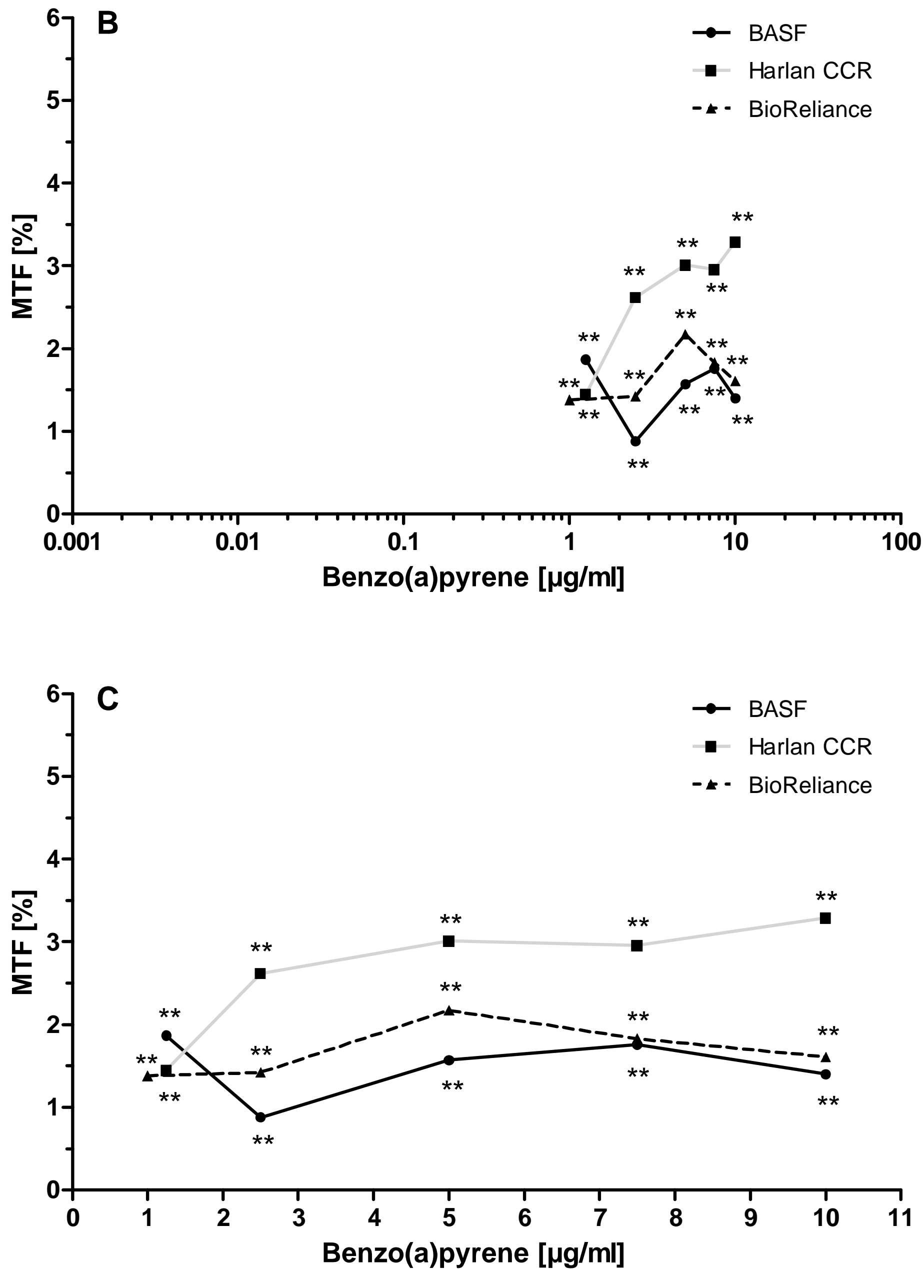


Figure 2: Morphological Transformation Frequency (MTF) shown as a percentage of transformed colonies compared to scorable colonies, for all laboratories testing coded benzo(a)pyrene: University of Metz repeated the experiment three times (**A**), whereas BASF, Harlan CCR and BioReliance performed a single experiment (**B**). For a better visualisation of the data, results from BASF, Harlan CCR and BioReliance were also represented with a linear scale (**C**)
** = $p < 0.01$ (one-sided Fisher's exact test)

4.1.7 Acceptance criteria and assessment

All acceptance criteria were met in all experiments performed by the four laboratories. These are summarised in Table 7.

Table 7: Acceptance criteria and assessment of benzo(a)pyrene results

Benzo(a)pyrene						
Criteria	**Laboratory**					
	Uni Metz			**BASF**	**Harlan CCR**	**BioReliance**
	TA1	**TA2**	**TA3**			
Number of scorable colonies per treatment group • 1000 or <1000 if positive result	**yes** (• 1018)	**yes** (• 1100)	**yes** (• 1174)	**yes** (• 1018)	**yes** (• 1896)	**yes** (• 1203)
Average number of 25-45 colonies per dish or >45 colonies if positive result or <25 colonies if negative result	**yes** (27.2-39.6)	**yes** (27.6-39.0)	**yes** (29.6-38.9)	**yes** (26.0-33.1)	**yes** (47.4-60.7)	**yes** (30.1-38.1)
Plating Efficiency of vehicle control >20%	**yes** (26.4%)	**yes** (26.0%)	**yes** (25.8%)	**yes** (30.1%)	**yes** (53.2%)	**yes** (25.1%)
Morphological Transformation Frequency of vehicle control <0.6%	**yes** (0.38%)	**yes** (0.26%)	**yes** (0.45%)	**yes** (0.08%)	**yes** (0.34%)	**yes** (0.42%)
Number of scorable concentrations • 5	**yes** (6[#])	**yes** (7)	**yes** (7)	**yes** (5)	**yes** (5)	**yes** (5)
Fisher's test *p*-value of positive control <0.05	na	na	na	na	na	na
Fulfilment of all assay acceptance criteria	**YES**	**YES**	**YES**	**YES**	**YES**	**YES**
Assessment	**+**	**+**	**+**	**+**	**+**	**+**

na = not applicable
[#] results for 1 µg/ml were not taken into consideration. See section 4.1.1 for details.

4.2 Conclusion of the Validation Management Team on Module 2

The data generated by University of Metz in the three experiment repeats showed a good within-laboratory reproducibility of the results for coded benzo(a)pyrene. .

Although no dose-dependent cytotoxicity could be observed (due to insolubility of the test chemical at higher concentrations), benzo(a)pyrene induced a reproducible increase in MTF. Results from this phase of the study were in agreement with published data for benzo(a)pyrene (Rivedal and Sanner, 1980).

In addition, these results showed reproducibility for benzo(a)pyrene between the laboratories and over time (since the experiments of University of Metz were performed prior to the main study) and demonstrated that the method had been successfully transferred to all laboratories.

Overall, the VMT agreed that the data generated were satisfactory and reproducible.

5 Module 3: Transferability

5.1 General Aspects

In general, the proposed test method can be performed in a laboratory that is experienced in routine cell culture techniques. Thus, given the level of experience in general cell/tissue culture, such a laboratory furnished with the appropriate test protocol and supporting SOPs could be expected to effectively conduct the CTA.

General cell culture laboratory equipment and instruments are sufficient to perform the proposed test method. All supplies and reagents are readily available commercially. Access to facilities for the irradiation of feeder cells is necessary.

The preparation of primary cells is more laborious and requires the isolation of cells from pregnant Syrian hamsters and the evaluation for their suitability for use in this assay. However, primary SHE cells are commercially available, though, as with any such preparation, the cells would need to be tested for their suitability.

Scoring of transformed colonies is at the moment still done manually using the microscope though methods for automation are being worked on. Proper training is therefore essential to ensure uniform and objective scoring to the extent possible.

5.2 Training

The CTA requires personnel trained for general cell biology and cell culture techniques (*e.g.* aseptic operations). Such expertise is available in most if not all Quality Control tissue culture laboratories.

The operator should, in particular, be trained in the scoring of transformed colonies. The training requirements for a person to be competent in scoring the plates are quite rigorous.

Three of the laboratories participating to this prevalidation study (Harlan CCR, BioReliance and BASF) had also been involved in the prevalidation study of the CTA in SHE cells at pH 6.7 where they gained experience in working with these cells. However, it became clear that the scoring of colonies produced with the protocol at pH 7.0 was slightly different from that at pH 6.7. In order to ensure that all laboratories participating in this prevalidation study would use a harmonised protocol and would be able to score appropriately, a training was held at the University of Metz, France, in July 2008. Representatives from all laboratories involved participated in the training. Agreement was reached on criteria for scoring the plates, using dishes treated with both the vehicle and positive controls. Overall, the training was extremely useful for harmonising the procedures among the laboratories for the prevalidation experiments.

As part of this prevalidation exercise a photo catalogue was produced by the lead laboratory (University of Metz) with the aim of standardising the scoring. The catalogue includes pictures of both non-transformed and transformed colonies. Examples of clearly scorable colonies, recognisably transformed colonies, colonies with questionable or mixed morphology, as well as examples of altered colonies that should not be scored were included in the catalogue to obtain an overview of the different types of colonies that can be encountered during a CTA experiment. Such a catalogue was found to be very useful in establishing consistency in assessing colony morphology and for the scoring of the experiments performed to assess the between-laboratory reproducibility.

5.3 Conclusion of the Validation Management Team on Module 3

It should be noted that three of the laboratories (BASF, Harlan CCR and BioReliance) had no experience in conducting the SHE CTA at pH 7.0, prior to starting this prevalidation study.

Basic cell culture experience and training in the conduct and scoring of the assay are important. In addition, the photo catalogue produced was found to be very useful in establishing consistency in assessing colony morphology and for the scoring of the experiments performed to assess the between-laboratory reproducibility.

The VMT agreed on the success of the method transfer.

This was also subsequently confirmed by the satisfactory results for the between-laboratory reproducibility (sections 4 and 6 on within- and between-laboratory reproducibility, respectively).

6 Module 4: Between-laboratory reproducibility

For the between-laboratory reproducibility assessment, the same conditions (medium, serum, SHE cells, controls and protocol) used for the within-laboratory reproducibility assessment were maintained. The following coded chemicals were tested (Table 1): anthracene, 2,4-diaminotoluene, 3-methylcholanthrene, o-toluidine HCl and phthalic anhydride.

The results of the between-laboratory reproducibility are summarised by chemical and laboratory. The data are shown in tables for each individual laboratory. Each table includes information on the total number of scorable colonies, RPE, colony density and size, the number of transformed colonies, MTF, and the result of the Fisher's exact test (*p*-value), for VC, test chemical at different concentrations and the PC.

An initial DRF test was performed by all laboratories to determine the experimental doses to be used in the TA, for each chemical (Figure 3, Figure 6, Figure 9, Figure 12 and Figure 15). Subsequently, the complete TA including concurrent cytotoxicity tests (Figure 4, Figure 7, Figure 10, Figure 13 and Figure 16) and MTF assay were performed (Figure 5, Figure 8, Figure 11, Figure 14 and Figure 17).

The statistical analysis was performed by the laboratories and recalculated by the statistician. The laboratories and the statistician conclusions showed complete concordance, which can also be considered as a data quality control check.

6.1 Anthracene

6.1.1 Dose-range finding test

Figure 3 shows the results of the DRF tests with anthracene. The common range of concentrations for DRF was 0.6-13 µg/ml. At the concentrations tested, limited cytotoxicity (IC_{80}) was observed by Harlan CCR, whereas the other laboratories saw some cell proliferation.

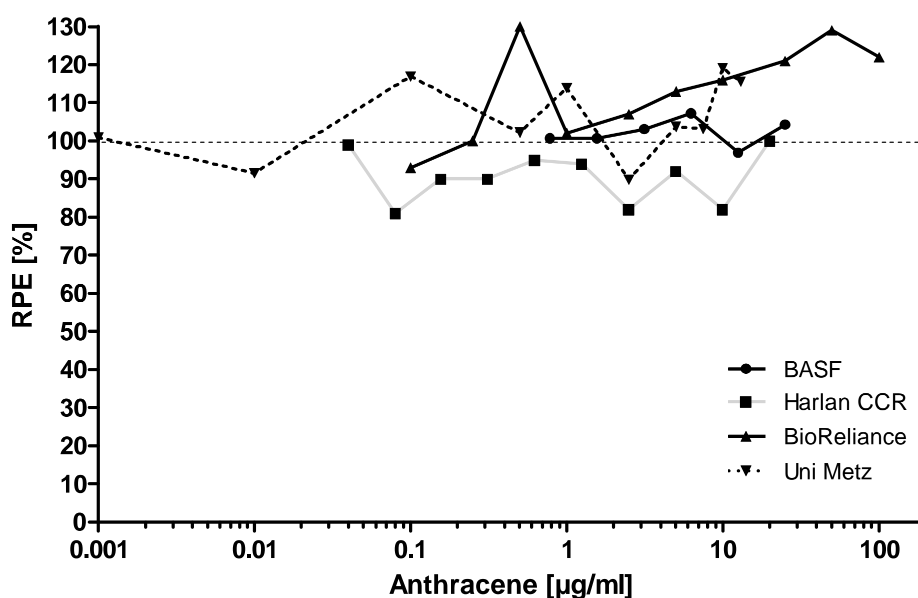


Figure 3: Relative plating efficiency (RPE) compared to vehicle control in all laboratories testing coded anthracene, for the Dose-Range Finding

6.1.2 Transformation Assay

VCs gave transformation frequencies within the expected range (• 0.6%): 0.40% (University of Metz), 0.25% (BASF), 0.27% (Harlan CCR) and 0.21% (BioReliance). The PC chemical benzo(a)pyrene led to the expected increase in morphologically transformed colonies: 2.85% (University of Metz), 1.36% (BASF), 1.85% (Harlan CCR) and 1.03% (BioReliance). It can be seen from Figure 4 that anthracene was not cytotoxic in any laboratory, nor did it induce a statistically significant increase in morphological transformation (Figure 5).

6.1.2.1 University of Metz

Anthracene was dissolved in DMSO. 13 µg/ml was selected as the top dose on the basis of the DRF test and based on the solubility of the chemical. The MTF values of the test chemical doses ranged from 0.24% to 0.89%. None of the test chemical concentrations induced a significant increase in MTF compared to the VC (*p* • 0.05). The results evaluated by University of Metz are shown in Table 8.

Table 8: Transformation assay results from University of Metz, testing coded anthracene

Uni Metz Anthracene (µg/ml)	Scorable colonies	RPE (%)	Colony density/size	Transformed colonies	MTF (%)	Fisher's exact test *p*-value (one-sided)
VC (DMSO 0.2%)	1238	100.0	+	5	0.40	-
1.0	1206	96.6	+	10	0.83	0.1384
2.5	1280	103.0	+	7	0.55	0.4097
5.0	1272	101.8	+	3	0.24	0.3483
7.5[#]	1330	106.7	+	9	0.68	0.2528
10[##]	1344	108.5	+	12	0.89	0.09724
13[##]	1390	111.9	+	7	0.50	0.778
PC	1193	96.0	+	34	2.85	<0.0001**

[#] = a slight opalescence was observed in the premix and the test medium, [##] = opalescence was observed in the premix and the test medium

VC = Vehicle Control, PC = Positive Control, RPE = Relative Plating Efficiency, MTF = Morphological Transformation Frequency, + = normal density/size.

** = $p < 0.01$ (one-sided Fisher's exact test)

6.1.2.2 BASF

Anthracene was dissolved in DMSO. 25 µg/ml was selected as the top dose on the basis of the DRF test and based on the solubility of the chemical. The MTF values of the test chemical doses ranged from 0.00% to 0.25%. None of the test chemical concentrations induced a significant increase in MTF compared to the VC (*p* • 0.05). The results evaluated by BASF are shown in Table 9.

Table 9: Transformation assay results from BASF, testing coded anthracene

BASF Anthracene (µg/ml)	Scorable colonies	RPE (%)	Colony density/size	Transformed colonies	MTF (%)	Fisher's exact test *p*-value (one-sided)
VC (DMSO 0.2%)	1575	100.0	+	4	0.25	-
2.5	1607	100.1	+	0	0.00	1.000
5.0	1569	96.7	+	4	0.25	0.635
10.0	1696	105.0	+	3	0.18	0.803
15.0	1731	107.3	+	3	0.17	0.811
20.0	1686	105.4	+	4	0.24	0.673
25.0	1606	99.0	+	4	0.25	0.648
PC	735	82.9	+	10	1.36	0.003**

VC = Vehicle Control, PC = Positive Control, RPE = Relative Plating Efficiency, MTF = Morphological Transformation Frequency, + = normal density/size.

** = $p < 0.01$ (one-sided Fisher's exact test)

6.1.2.3 Harlan CCR

Anthracene was dissolved in DMSO. 20 µg/ml was selected as the top dose on the basis of the DRF test and based on the solubility of the chemical. The MTF values of the test chemical doses ranged from 0.18% to 0.26%. None of the test chemical concentrations induced a significant increase in MTF compared to the VC (*p* • 0.05). The results evaluated by Harlan CCR are shown in Table 10.

Table 10: Transformation assay results from Harlan CCR, testing coded anthracene

Harlan CCR Anthracene (µg/ml)	Scorable colonies	RPE (%)	Colony density/size	Transformed colonies	MTF (%)	Fisher's exact test *p*-value (one-sided)
VC (DMSO 0.2%)	1498	100.0	+	4	0.27	-
0.63	1418	94.6	+	3	0.21	>0.5
1.25	1524	101.7	+	3	0.20	>0.5
2.5	1546	103.2	+	4	0.26	>0.5
5.0	1438	96.1	+	3	0.21	>0.5
10.0	1556	103.9	+	4	0.26	>0.5
20.0	1626	108.5	+	3	0.18	>0.5
PC	1458	97.5	+	27	1.85	<0.001**

VC = Vehicle Control, PC = Positive Control, RPE = Relative Plating Efficiency, MTF = Morphological Transformation Frequency, + = normal density/size.
** = $p < 0.01$ (one-sided Fisher's exact test)

6.1.2.4 BioReliance

Anthracene was dissolved in DMSO. 100 µg/ml was selected as the top dose on the basis of the DRF test and based on the solubility of the chemical. The MTF values of the test chemical doses ranged from 0.20% to 0.36%. None of the test chemical concentrations induced a significant increase in MTF compared to the VC (*p* • 0.05). The results evaluated by BioReliance are shown in Table 11.

Table 11: Transformation assay results from BioReliance, testing coded anthracene

BioReliance Anthracene (µg/ml)	Scorable colonies	RPE (%)	Colony density/size	Transformed colonies	MTF (%)	Fisher's exact test *p*-value (one-sided)
VC (DMSO 0.2%)	1463	100.0	+	3	0.21	-
2.5	1391	95.6	+	5	0.36	0.336
5.0	1331	92.0	+	3	0.23	0.611
10	1532	105.3	+	3	0.20	0.678
25	1474	101.0	+	4	0.27	0.504
50	1396	96.1	+	4	0.29	0.474
100	1512	103.9	+	4	0.26	0.518
PC	1546	105.7	+	16	1.03	0.003**

VC = Vehicle Control, PC = Positive Control, RPE = Relative Plating Efficiency, MTF = Morphological Transformation Frequency, + = normal density/size.
** = $p < 0.01$ (one-sided Fisher's exact test)

6.1.2.5 Concurrent cytotoxicity (Relative Plating Efficiency)

Cytotoxicity of anthracene was evaluated by RPE assessment in all laboratories (Figure 4). The common range tested was 2.5-40 µg/ml. No evident signs of cytotoxicity were observed by any laboratory.

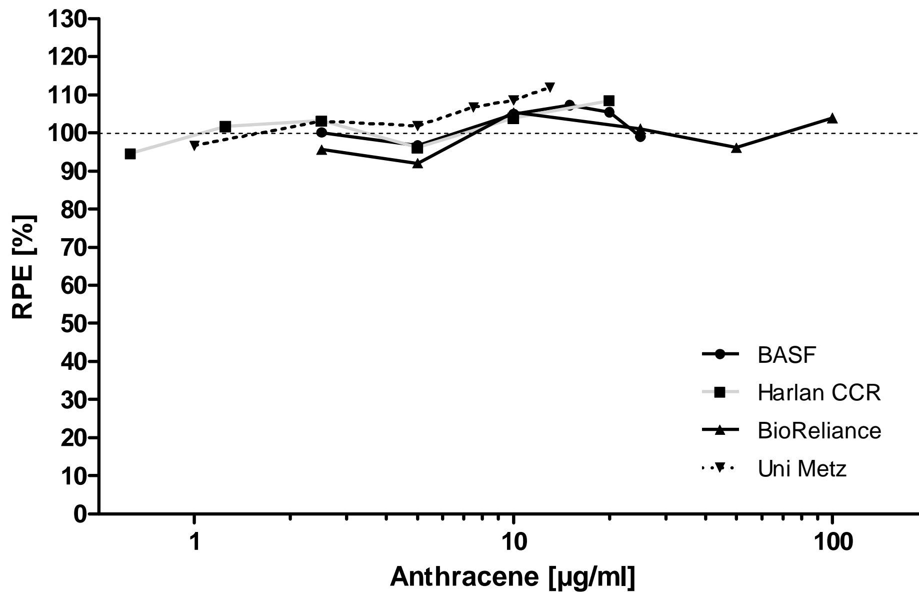


Figure 4: Relative plating efficiency (RPE) compared to vehicle control in all laboratories testing coded anthracene

6.1.2.6 Morphological transformation frequency

MTF results with anthracene are shown in Figure 5. Anthracene did not induce a statistically significant increase in morphological transformation in any of the laboratories.

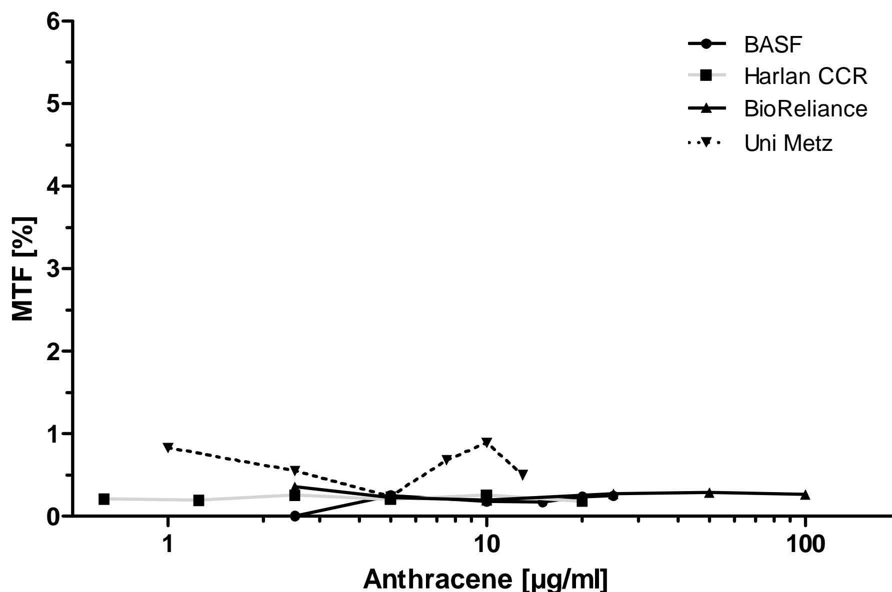


Figure 5: Morphological Transformation Frequency (MTF) shown as a percentage of transformed colonies compared to total colonies, for all laboratories testing anthracene

6.1.2.7 Acceptance criteria and assessment

All acceptance criteria were met in all laboratories. Anthracene was assessed to be negative by all laboratories (Table 12).

Table 12: Acceptance criteria and assessment of anthracene results

Anthracene				
Criteria	**Laboratory**			
	BASF	**Harlan CCR**	**BioReliance**	**Uni Metz**
Number of scorable colonies per treatment group • 1000 or <1000 if positive result	**yes** (• 1569,735[#])	**yes** (• 1418)	**yes** (• 1331)	**yes** (• 1193)
Average number of 25-45 colonies per dish or >45 colonies if positive result or <25 colonies if negative result	**yes** (33.6-43.5)	**yes** (34.6-40.7)	**yes** (33.7-38.7)	**yes** (30.0-35.0)
Plating Efficiency of vehicle control >20%	**yes** (36.9%)	**yes** (57.7%)	**yes** (30.5%	**yes** (20.9%)
Morphological Transformation Frequency of vehicle control <0.6%	**yes** (0.25%)	**yes** (0.27%)	**yes** (0.21%)	**yes** (0.40%)
Number of scorable concentrations • 5	**yes** (6)	**yes** (6)	**yes** (6)	**yes** (6)
Fisher's test *p*-value of positive control <0.05	**yes** (0.003)	**yes** (<0.001)	**yes** (0.003)	**yes** (<0.0001)
Fulfilment of all assay acceptance criteria	**YES**	**YES**	**YES**	**YES**
Assessment	**-**	**-**	**-**	**-**

[#] 735 colonies for the positive control

6.1.3 Conclusion

Treatment with anthracene did not produce a statistically significant increase in morphologically transformed colonies in any laboratory at any dose tested. As such, anthracene is considered to be non-transforming in the SHE pH 7.0 CTA. These results agree with published data (Heidelberger *et al.*, 1983; Tennant *et al.*, 1985).

6.2 2,4-Diaminotoluene

6.2.1 Dose-range finding test

Figure 6 shows the results of the DRF tests with 2,4-diaminotoluene. All laboratories observed a dose-dependent cytotoxicity induced by 2,4-diaminotoluene at the concentrations tested.

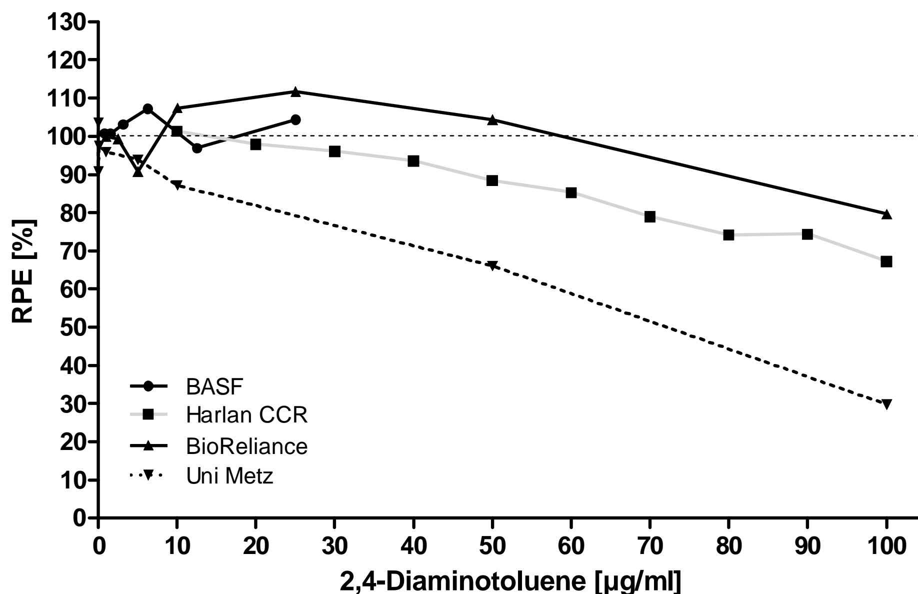


Figure 6: Relative plating efficiency (RPE) compared to vehicle control in all laboratories testing coded 2,4-diaminotoluene, for the Dose-Range Finding

6.2.2 Transformation assay

VCs gave transformation frequencies within the expected range (• 0.6%): 0.39% (University of Metz), 0.28% (BASF), 0.20% (Harlan CCR) and 0.21% (BioReliance). The PC chemical benzo(a)pyrene led to the expected increase in morphologically transformed colonies: 1.82% (University of Metz), 1.70% (BASF), 2.34% (Harlan CCR) and 1.03% (BioReliance).

6.2.2.1 University of Metz

2,4-Diaminotoluene was dissolved in DMSO. The concentrations were selected on the basis of the DRF test. The MTF values of the test chemical doses ranged from 1.03% to 3.41% and the VC value was 0.39%. Three test chemical concentrations induced a significant increase in MTF compared to the VC ($p < 0.05$) (Table 13). At the two highest concentrations no significant increase in MTF was observed, most probably due to the cytotoxicity caused by the test chemical. 50 and 75 µg/ml concentrations were not considered scorable because the total number of colonies was below the limit (<1000) with a negative result.

Table 13 : Transformation assay results from University of Metz, testing coded 2,4-diaminotoluene

Uni Metz 2,4-diaminotoluene (µg/ml)	Scorable colonies	RPE (%)	Colony density/size	Transformed colonies	MTF (%)	Fisher's exact test *p*-value (one-sided)
VC (DMSO 0.2%)	1291	100.0	+	5	0.39	-
0.1	1159	89.6	+	14	1.21	0.01794*
1	1218	94.2	+	38	3.12	<0.0001**
5	1189	94.6	+	28	2.35	<0.0001**
10	1257	98.1	+	35	2.78	<0.0001**
25	1230	84.7	+	42	3.41	<0.0001**
50	967	56.9	+++	10	1.03	0.0543
75	170	9.9	+++	3	1.76	0.055
PC	1265	98.3	+	23	1.82	0.0003**

VC = Vehicle Control, PC = Positive Control, RPE = Relative Plating Efficiency, MTF = Morphological Transformation Frequency, + = normal density/size, ++ = slightly reduced density/size, +++ = highly reduced density/size.
* = $p < 0.05$, ** = $p < 0.01$ (one-sided Fisher's exact test)

6.2.2.2 BASF

The first experiment (TA1) did not fulfill the acceptance criterion for the number of scorable concentrations and the number of transformed colonies for one test chemical concentration (10 µg/ml) was in the same range as for the PC (see section 12.3 for details). BASF was thus asked by the VMT to repeat the experiment (TA2).
2,4-Diaminotoluene was dissolved in DMSO. The concentrations were selected on the basis of the DRF test. The MTF values of the test chemical doses ranged from 0.85% to 2.70% and the VC value was 0.28%. All test chemical concentrations, except the lowest, induced a significant increase in MTF compared to the VC ($p < 0.05$) (Table 14).

Table 14: Transformation assay TA2 results from BASF, testing coded 2,4-diaminotoluene

BASF 2,4-diaminotoluene (µg/ml) TA2	Scorable colonies	RPE (%)	Colony density/size	Transformed colonies	MTF (%)	Fisher's exact test *p*-value (one-sided)
VC (DMSO 0.2%)	1080	100.0	+	3	0.28	-
5	1173	108.6	+	10	0.85	0.061
10	1116	103.3	+	12	1.08	0.002**
20	1080	100.0	+	10	0.93	0.045*
30	1075	99.5	+	29	2.70	<0.001**
40	1091	67.3	++	16	1.47	0.002**
PC	1178	109.2	+	20	1.70	<0.001**

VC = Vehicle Control, PC = Positive Control, RPE = Relative Plating Efficiency, MTF = Morphological Transformation Frequency, + = normal density/size, ++ = slightly reduced density/size.
* = $p < 0.05$, ** = $p < 0.01$ (one-sided Fisher's exact test)

6.2.2.3 Harlan CCR

2,4-Diaminotoluene was dissolved in DMSO. The concentrations were selected on the basis of the DRF test. The MTF values of the test chemical doses ranged from 0.87% to 3.51% and the VC value was 0.20%. All test chemical concentrations induced a significant increase in MTF compared to the VC ($p < 0.05$) (Table 15).

Table 15: Transformation assay results from Harlan CCR, testing coded 2,4-diaminotoluene

Harlan CCR 2,4-diaminotoluene (µg/ml)	Scorable colonies	RPE (%)	Colony density/size	Transformed colonies	MTF (%)	Fisher's exact test *p*-value (one-sided)
VC (DMSO 0.2%)	1519	100.0	+	3	0.20	-
20.0	1729	113.9	+	15	0.87	0.008**
40.0	1469	96.7	+	31	2.11	<0.001**
60.0	1168	79.0	+	41	3.51	<0.001**
80.0	1202	78.2	++	31	2.58	<0.001**
100.0	1009	51.3	+++	23	2.28	<0.001**
PC	1625	107.1	+	38	2.34	<0.001**

VC = Vehicle Control, PC = Positive Control, RPE = Relative Plating Efficiency, MTF = Morphological Transformation Frequency, + = normal density/size, ++ = slightly reduced density/size, +++ = highly reduced density/size.
** = $p < 0.01$ (one-sided Fisher's exact test)

6.2.2.4 BioReliance

2,4-Diaminotoluene was dissolved in DMSO. The concentrations were selected on the basis of the DRF test. The MTF values of the test chemical doses ranged from 0.10% to 0.93% and the VC value was 0.21%. Three test chemical concentrations induced a significant increase in MTF compared to the VC ($p < 0.05$). At the highest concentration no significant increase in MTF was observed, most probably due to the cytotoxicity caused by the test chemical (Table 16).

Table 16: Transformation assay results from BioReliance, testing coded 2,4-diaminotoluene

BioReliance 2,4-diaminotoluene (µg/ml)	Scorable colonies	RPE (%)	Colony density/size	Transformed colonies	MTF (%)	Fisher's exact test *p*-value (one-sided)
VC (DMSO 0.2%)	1463	100.0	+	3	0.21	-
5.0	1720	117.6	+	13	0.76	0.023*
10	1759	120.3	+	13	0.74	0.026*
25	1781	121.9	+	10	0.56	0.091
50	1609	110.3	+	15	0.93	0.007**
100	1004	68.6	+++	1	0.10	0.877
PC	1546	105.7	+	16	1.03	0.003**

VC = Vehicle Control, PC = Positive Control, RPE = Relative Plating Efficiency, MTF = Morphological Transformation Frequency, + = normal density/size, +++ = highly reduced density/size.
* = $p < 0.05$, ** = $p < 0.01$ (one-sided Fisher's exact test)

6.2.2.5 Concurrent cytotoxicity (Relative Plating Efficiency)

Cytotoxicity of 2,4-diaminotoluene was evaluated by RPE assessment (Figure 7). The common range tested by all laboratories was 20-40 µg/ml and 2,4-diaminotoluene was shown to be dose-dependently cytotoxic in all laboratories. Although the shapes of the curves were similar in all laboratories, the chemical resulted more toxic in the BASF and University of Metz laboratories.

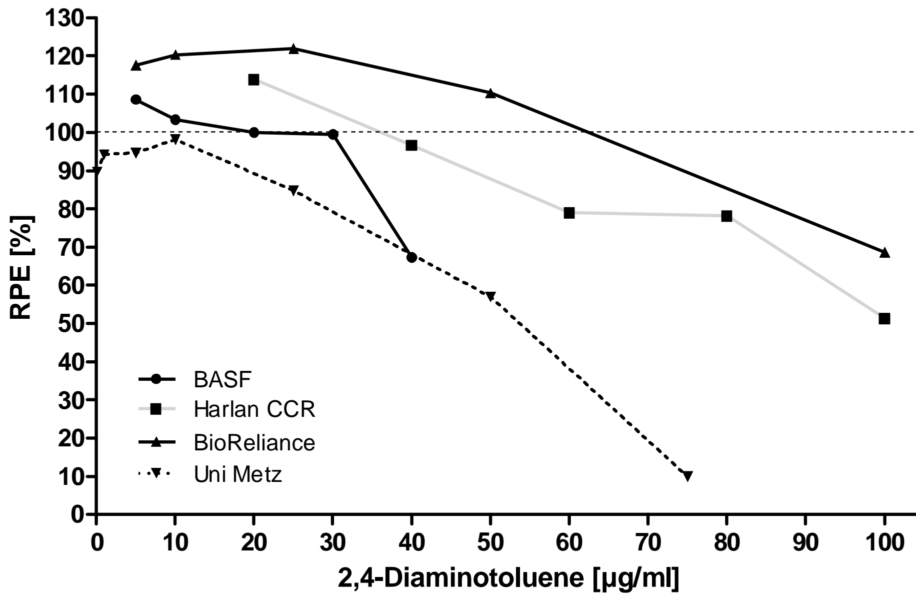


Figure 7: Relative plating efficiency (RPE) compared to vehicle control in all laboratories testing coded 2,4-diaminotoluene

6.2.2.6 Morphological transformation frequency

MTF results with 2,4-diaminotoluene are shown in Figure 8. 2,4-Diaminotoluene induced a statistically significant increase in morphological transformation compared to the VC in all laboratories.

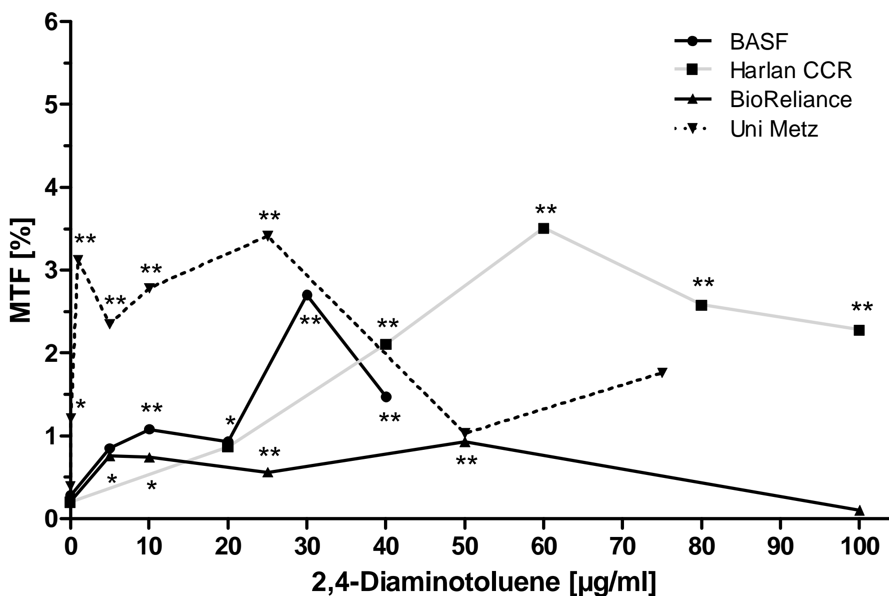


Figure 8: Morphological Transformation Frequency (MTF) shown as a percentage of transformed colonies compared to total colonies for all laboratories testing coded 2,4-diaminotoluene

* = $p < 0.05$, ** = $p < 0.01$ (one-sided Fisher's exact test)

6.2.2.7 Acceptance criteria and assessment

All acceptance criteria were met in all laboratories. 2,4-diaminotoluene was assessed to be positive by all laboratories (Table 17).

Table 17: Acceptance criteria and assessment of 2,4-diaminotoluene results

2,4-Diaminotoluene				
Criteria	**Laboratory**			
	Uni Metz	**BASF (TA2)**	**Harlan CCR**	**BioReliance**
Number of scorable colonies per treatment group• 1000 or <1000 if positive result	**yes** (• 1159)	**yes** (• 1075)	**yes** (• 1009)	**yes** (• 1004)
Average number of 25-45 colonies per dish or >45 colonies if positive result or <25 colonies if negative result	**yes** (29.1-32.4)	**yes** (26.9-29.5)	**yes** (26.0-43.3)	**yes** (25.1-44.6)
Plating Efficiency of vehicle control >20%	**yes** (21.6%)	**yes** (24.5%)	**yes** (50.7%)	**yes** (30.5%)
Morphological Transformation Frequency of vehicle control <0.6%	**yes** (0.39%)	**yes** (0.28%)	**yes** (0.20%)	**yes** (0.21%)
Number of scorable concentrations • 5	**yes** (5[#])	**yes** (5)	**yes** (5)	**yes** (5)
Fisher's test *p*-value of positive control <0.05	**yes** (<0.0003)	**yes** (<0.001)	**yes** (<0.001)	**yes** (0.003)
Fulfilment of all assay acceptance criteria	**YES**	**YES**	**YES**	**YES**
Assessment	**+**	**+**	**+**	**+**

[#] results for 50 and 75 µg/ml were not taken into consideration. See section 6.2.2.1for details.

6.2.3 Conclusion

Treatment with 2,4-diaminotoluene produced a statistically significant increase in morphologically transformed colonies in all laboratories. As such, 2,4-diaminotoluene was considered to be a positive transforming agent in the SHE pH 7.0 CTA. These results agree with published data (Holen *et al.*, 1990).

6.3 3-Methylcholanthrene

6.3.1 Dose-range finding test

Figure 9 shows the results of the DRF tests with 3-methylcholanthrene.

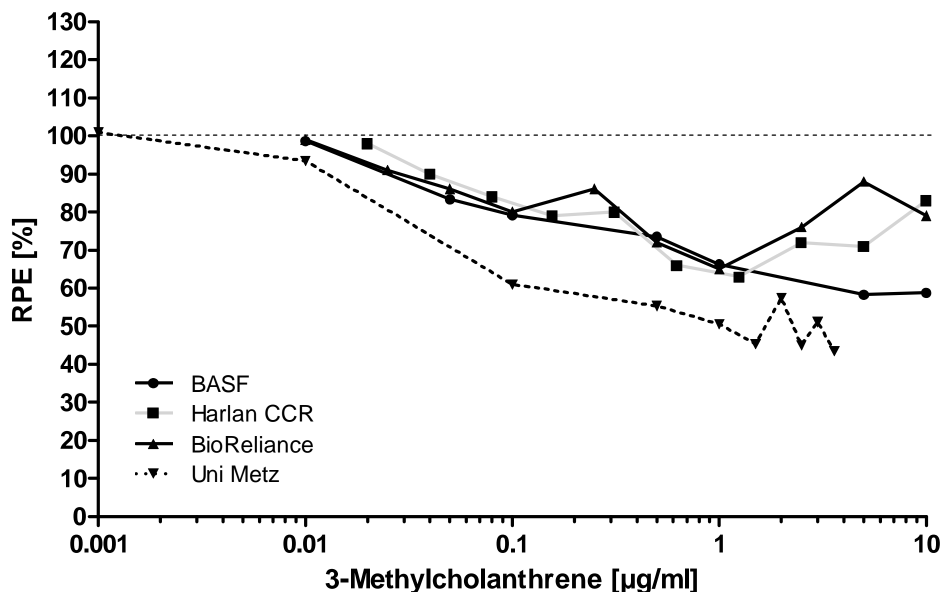


Figure 9: Relative plating efficiency (RPE) compared to vehicle control in all laboratories testing coded 3-methylcholanthrene, for Dose-Range Finding

6.3.2 Transformation assay

VCs gave transformation frequencies within the expected range (• 0.6%): 0.51% (University of Metz), 0.25% (BASF), 0.32% (Harlan CCR), and 0.38% (BioReliance). The PC chemical benzo(a)pyrene led to the expected increase in morphologically transformed colonies: 2.46% (University of Metz), 1.36% (BASF), 2.04% (Harlan CCR), and 1.59% (BioReliance). Figure 10 shows that 3-methylcholanthrene was not cytotoxic at the doses tested by Harlan CCR and BioReliance whereas some cytotoxicity was observed in the other two laboratories. Despite the differences in cytotoxicity, all laboratories showed 3-methylcholanthrene to induce a statistically significant increase in morphological transformation at all doses tested (Figure 11).

6.3.2.1 University of Metz

3-Methylcholanthrene was dissolved in DMSO. The concentrations were selected on the basis of the dose range suggested by the VMT and the DRF experiment, and the results evaluated by University of Metz are shown in Table 18. The MTF values of the test chemical doses ranged from 0.77% to 1.43%. Four test chemical concentrations induced significant increases in MTF compared to the VC ($p < 0.05$).
The three highest test chemical concentrations were not considered valid because the average number of colonies per dish was below the limit for positive results (<25 colonies) for 0.1 and 1 µg/ml (20.9 and 24.4 colonies per dish, respectively), and because the total number of colonies for 2 µg/ml was below the limit (<1000) with a negative result.

Table 18: Transformation assay results from University of Metz, testing coded 3-methylcholanthrene

Uni Metz 3-methylcholanthrene (µg/ml)	Scorable colonies	RPE (%)	Colony density/size	Transformed colonies	MTF (%)	Fisher's exact test *p*-value (one-sided)
VC (DMSO 0.2%)	1373	100.0	+	7	0.51	-
0.0005	1445	105.1	+	13	0.90	0.156
0.001	1332	96.8	+	13	0.98	0.116
0.005	1117	85.6	+	15	1.34	0.023 *
0.01	1099	71.4	++	15	1.36	0.021 *
0.1	976	63.3	++	14	1.43	0.017*
1	796	49.2	++	11	1.38	0.030*
2	775	45.7	++	6	0.77	0.312
PC	1344	100.5	+++	33	2.46	<0.0001**

VC = Vehicle Control, PC = Positive Control, RPE = Relative Plating Efficiency, MTF = Morphological Transformation Frequency, + = normal density/size, ++ = slightly reduced density/size, +++ = highly reduced density/size.
* = $p < 0.05$, ** = $p < 0.01$ (one-sided Fisher's exact test)

6.3.2.2 BASF

3-Methylcholanthrene was dissolved in DMSO. The concentrations were selected on the basis of the dose range suggested by the VMT and the DRF experiment, and the results evaluated by BASF are shown in Table 19. The MTF values of the test chemical doses ranged from 0.38% to 1.14%. Three test chemical concentrations induced significant increases in MTF compared to the VC ($p < 0.05$).

Table 19: Transformation assay results from BASF, testing coded 3-methylcholanthrene

BASF 3-methylcholanthrene (µg/ml)	Scorable colonies	RPE (%)	Colony density/size	Transformed colonies	MTF (%)	Fisher's exact test *p*-value (one-sided)
VC (DMSO 0.2%)	1575	100.0	+	4	0.25	-
0.31	1041	66.7	+	4	0.38	0.401
0.63	1001	65.4	+	4	0.40	0.379
1.25	1054	72.5	+	12	1.14	0.005**
2.50	1486	64.3	+	12	0.81	0.029*
5.00	1582	66.2	+	6	0.38	0.379
10.00	1634	68.5	+	15	0.92	0.012*
PC	735	82.9	+	10	1.36	0.003**

VC = Vehicle Control, PC = Positive Control, RPE = Relative Plating Efficiency, MTF = Morphological Transformation Frequency, + = normal density/size.
* = $p < 0.05$, ** = $p < 0.01$ (one-sided Fisher's exact test)

BASF used for this experiment the same VC and PC that were used to test anthracene.

6.3.2.3 Harlan CCR

3-Methylcholanthrene was dissolved in DMSO. The concentrations were selected on the basis of the dose range suggested by the VMT and the DRF experiment, and the results evaluated by Harlan CCR are shown in Table 20. The MTF values of the test chemical doses ranged from 1.12% to 2.99%. All test chemical concentrations induced significant increases in MTF compared to the VC ($p < 0.05$), with a dose-dependent effect.

Table 20: Transformation assay results from Harlan CCR, testing coded 3-methylcholanthrene

Harlan CCR 3-methylcholanthrene (µg/ml)	Scorable colonies	RPE (%)	Colony density/size	Transformed colonies	MTF (%)	Fisher's exact test *p*-value (one-sided)
VC (DMSO 0.2%)	1565	100.0	+	5	0.32	-
0.16	1345	86.1	+	15	1.12	<0.0005**
0.31	1438	92.1	+	22	1.53	<0.0005**
0.63	1515	95.9	+	20	1.32	<0.0005**
1.25	1428	91.4	+	26	1.82	<0.0005**
2.5	1551	97.6	+	42	2.71	<0.0005**
5.0	1407	89.9	+	42	2.99	<0.0005**
10.0	1569	99.3	+	39	2.49	<0.0005**
PC	1470	64.2	+	30	2.04	<0.0005**

VC = Vehicle Control, PC = Positive Control, RPE = Relative Plating Efficiency, MTF = Morphological Transformation Frequency, + = normal density/size.
** = $p < 0.01$ (one-sided Fisher's exact test)

6.3.2.4 BioReliance

3-Methylcholanthrene was dissolved in DMSO. The concentrations were selected on the basis of the dose range suggested by the VMT and the DRF experiment, and the results evaluated by BioReliance are shown in Table 21. The MTF values of the test chemical doses ranged from 1.29% to 2.24%. All test chemical concentrations induced significant increases in MTF compared to the VC ($p < 0.05$).

Table 21: Transformation assay results from BioReliance, testing coded 3-methylcholanthrene

BioReliance 3-methylcholanthrene (µg/ml)	Scorable colonies	RPE (%)	Colony density/size	Transformed colonies	MTF (%)	Fisher's exact test *p*-value (one-sided)
VC (DMSO 0.2%)	1047	100.0	+	4	0.38	-
1.0	1008	96.4	+	18	1.79	0.002**
2.5	1009	96.7	+	22	2.18	<0.0005**
5.0	1005	96.3	+	16	1.59	0.004**
7.5	1025	98.8	+	23	2.24	<0.0005**
10	1008	96.6	+	13	1.29	0.020*
PC	1132	108.3	+	18	1.59	0.004**

VC = Vehicle Control, PC = Positive Control, RPE = Relative Plating Efficiency, MTF = Morphological Transformation Frequency, + = normal density/size.
* = $p < 0.05$, ** = $p < 0.01$ (one-sided Fisher's exact test)

6.3.2.5 Concurrent cytotoxicity (Relative Plating Efficiency)

Cytotoxicity of 3-methylcholanthrene was evaluated by RPE assessment in all laboratories (Figure 10). 3-Methylcholanthrene was not cytotoxic at the doses tested by Harlan CCR and BioReliance whereas some cytotoxicity was observed in the other two laboratories. University of Metz tested a wider dose range and a clear dose-dependent cytotoxicity curve can be seen.

BASF reported that the shift in the RPE was most probably due to the seeding procedure employed. This has been observed when several experiments were performed using a single cell preparation in one day, and the cells employed in later experiments were maintained in the medium for extended periods of time before seeding.

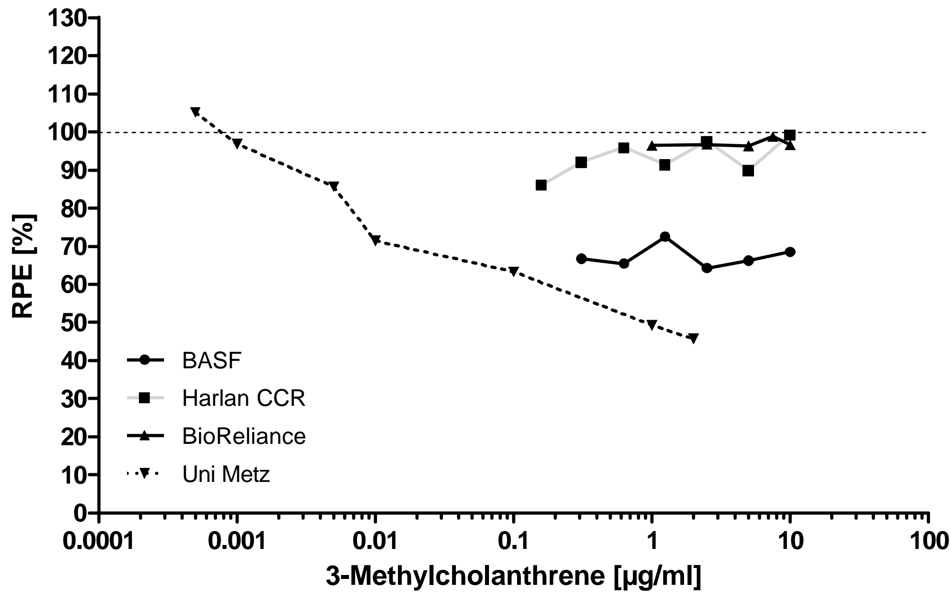


Figure 10: Relative plating efficiency (RPE) compared to vehicle control in all laboratories testing coded 3-methylcholanthrene

6.3.2.6 Morphological transformation frequency

MTF results with 3-methylcholanthrene are shown in Figure 11. The concentrations tested by all laboratories were in the range of 1-2 µg/ml. All laboratories showed 3-methylcholanthrene to induce a statistically significant increase in morphological transformation at most of if not all doses tested, with a clear dose-dependent response for Harlan CCR.

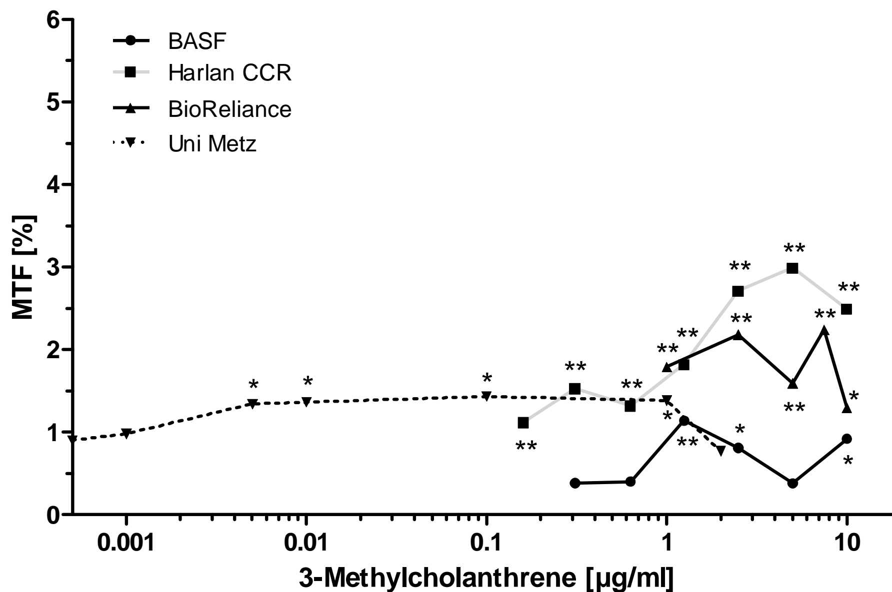


Figure 11: Morphological Transformation Frequency (MTF) shown as a percentage of transformed colonies compared to total colonies for all laboratories testing 3-methylcholanthrene.

* = $p < 0.05$, ** = $p < 0.01$ (one-sided Fisher's exact test)

6.3.2.7 Acceptance criteria and assessment

All acceptance criteria were met in all laboratories, except for University of Metz which failed to meet the criteria on the number of scorable concentrations (four instead of five). University of Metz showed clearly positive results (a statistically significant increase in MTF was observed at two valid concentrations), although only four concentrations fulfilled all the acceptance criteria. Therefore the VMT considered these results acceptable for the overall evaluation of the study.
3-methylcholanthrene was assessed to be positive by all laboratories (Table 22).

Table 22: Acceptance criteria and assessment of 3-methylcholanthrene results

3-Methylcholanthrene				
Criteria	**Laboratory**			
	Uni Metz	**BASF**	**Harlan CCR**	**BioReliance**
• 1000 scorable colonies per treatment group or <1000 if positive result	**yes** (• 1099)	**yes** (• 1001,735*)	**yes** (• 1345)	**yes** (• 1005)
Average number of 25-45 colonies per dish or >45 colonies if positive result or <25 colonies if negative result	**yes** (27.5-36.3)	**yes** (26.5-41.7)	**yes** (33.7-39.2)	**yes** (25.2-28.4)
Plating Efficiency of vehicle control >20%	**yes** (20.3%)	**yes** (36.9%	**yes** (52.2%	**yes** (21.8%)
Morphological Transformation Frequency of vehicle control <0.6%	**yes** (0.51%)	**yes** (0.25%)	**yes** (0.32%)	**yes** (0.38%)
Number of scorable concentrations • 5	**no** (4[#])	**yes** (6)	**yes** (7)	**yes** (5)
Fisher's test *p*-value of positive control <0.05	**yes** (<0.0001)	**yes** (0.003)	**yes** (<0.0005)	**yes** (0.004)
Fulfilment of all assay acceptance criteria	**NO[##]**	**YES**	**YES**	**YES**
Assessment	**+**	**+**	**+**	**+**

* 735 colonies for the positive control
[#] results for for 0.01, 0.1, and 2 µg/ml were not taken into consideration (not valid). See section 6.3.2.1 for details.
[##] considered acceptable for the overall study evaluation as there were 2 statistically significant positive concentrations.

6.3.3 Conclusion

Treatment with 3-methylcholanthrene produced a statistically significant increase in morphologically transformed colonies in all laboratories. As such, 3-methylcholanthrene was considered to be a positive transforming agent in the SHE pH 7.0 CTA. These results agree with published data (Heidelberger *et al.*, 1983).

6.4 O-toluidine HCl

6.4.1 Dose-Range finding test

Figure 12 shows the results of the DRF tests with o-toluidine HCl. Harlan CCR's results showed little cytotoxicity whereas the results from the other laboratories, having tested through a larger range, showed complete dose-dependent cytotoxicity curves.

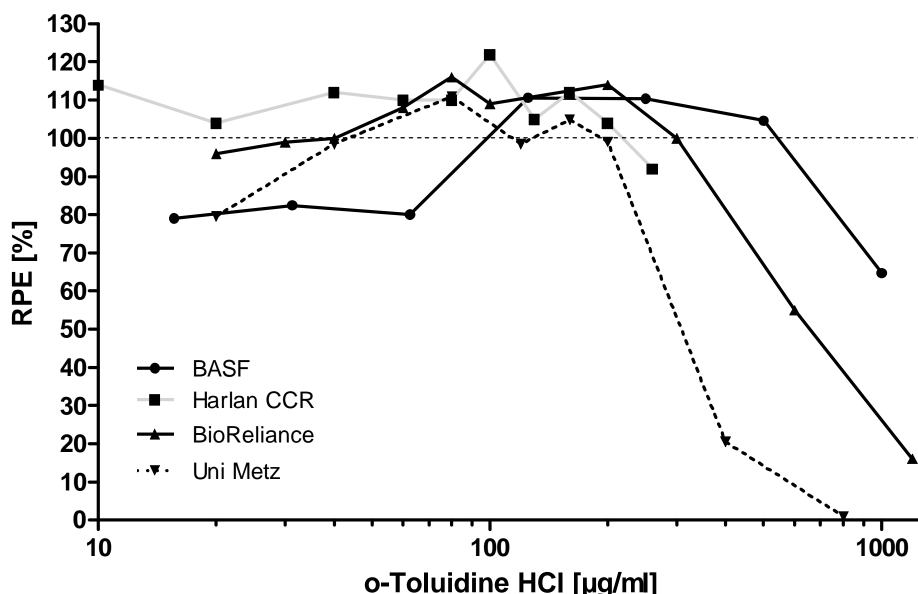


Figure 12: Relative plating efficiency compared to vehicle control in all laboratories testing coded o-toluidine HCl, for Dose-Range Finding

6.4.2 Transformation assay

VCs gave transformation frequencies within the expected range (• 0.6%): 0.49% (University of Metz), 0.26% (BASF), 0.35% (Harlan CCR), and 0.30% (BioReliance). The PC chemical benzo(a)pyrene led to the expected increase in morphologically transformed colonies: 3.02% (University of Metz), 1.43% (BASF), 2.29% (Harlan CCR) and 1.37% (BioReliance).

6.4.2.1 University of Metz

o-Toluidine HCl was dissolved in DMSO. The concentrations were selected on the basis of the suggested dose range by the VMT, from findings from solubility testing and the DRF test. The results evaluated by University of Metz are shown in Table 23. The MTF values of the test chemical doses ranged from 1.47% to 3.69%. All the test chemical concentrations induced statistically significant increases in MTF compared to the VC ($p < 0.01$).

Table 23: Transformation assay results from University of Metz, testing coded o-toluidine HCl

Uni Metz o-toluidine HCl (µg/ml)	Scorable colonies	RPE (%)	Colony density/size	Transformed colonies	MTF (%)	Fisher's exact test *p*-value (one-sided)
VC (DMSO 0.2%)	1416	100.0	+	7	0.49	-
20	1192	84.0	+	44	3.69	<0.001**
40	1272	89.6	+	47	3.69	<0.001**
80	1268	91.0	+	20	1.58	0.004**
120	1155	83.4	+	17	1.47	0.009**
160	1215	85.8	+	26	2.14	<0.001**
300	1180	83.7	+	35	2.97	<0.001**
PC	1358	95.7	+	41	3.02	<0.001**

VC = Vehicle Control, PC = Positive Control, RPE = Relative Plating Efficiency, MTF = Morphological Transformation Frequency, + = normal density/size.
** = $p < 0.01$ (one-sided Fisher's exact test)

6.4.2.2 BASF

o-Toluidine HCl was dissolved in DMSO. The concentrations were selected on the basis of the suggested dose range by the VMT, from findings from solubility testing and the DRF test. The results evaluated by BASF are show in Table 24. The MTF values of the test chemical doses ranged from 0.42% to 1.54%. All the test chemical concentrations dose-dependently induced statistically significant increases in MTF compared to the VC ($p < 0.01$).
Results for 200 and 400 µg/ml were not taken into consideration since the average number of colonies per dish was beyond the limit for a negative result (>45).

Table 24: Transformation assay results from BASF, testing coded o-toluidine HCl

BASF o-toluidine HCl (µg/ml)	Scorable colonies	RPE (%)	Colony density/size	Transformed colonies	MTF (%)	Fisher's exact test *p*-value (one-sided)
VC (DMSO 0.2%)	1524	100.0	+	4	0.26	-
100	-	-	-	-	-	-
200	2123	139.1	+	9	0.42	0.305
400	2109	139.8	+	9	0.43	0.301
600	1879	124.2	+	17	0.90	0.013*
800	1649	110.8	++	25	1.52	<0.001**
1 000	1752	77.9	++	27	1.54	<0.001**
PC	1396	92.0	+	20	1.43	<0.001**

VC = Vehicle Control, PC = Positive Control, RPE = Relative Plating Efficiency, MTF = Morphological Transformation Frequency, + = normal density/size, ++ = slightly reduced density/size.
* = $p < 0.05$, ** = $p < 0.01$ (one-sided Fisher's exact test)

6.4.2.3 Harlan CCR

o-Toluidine HCl was dissolved in DMSO. The concentrations were selected on the basis of the suggested dose range by the VMT, from findings from solubility testing and the DRF test. The results evaluated by Harlan CCR are shown in Table 25. The MTF values of the test chemical doses ranged from 0.91% to 2.54%. All the test chemical concentrations induced statistically significant increases in MTF compared to the VC ($p < 0.01$), with a dose-dependent effect.

Table 25: Transformation assay results from Harlan CCR, testing coded o-toluidine HCl

Harlan CCR o-toluidine HCl (µg/ml)	Scorable colonies	RPE (%)	Colony density/size	Transformed colonies	MTF (%)	Fisher's exact test *p*-value (one-sided)
VC (DMSO 0.2%)	1442	100.0	+	5	0.35	-
80.0	1323	91.8	+	12	0.91	<0.001**
100.0	1471	102.0	+	14	0.95	<0.001**
130.0	1425	97.6	+	19	1.33	<0.001**
160.0	1321	91.8	+	23	1.74	<0.001**
200.0	1338	91.6	+	31	2.32	<0.001**
260.0	1494	103.6	+	38	2.54	<0.001**
PC	1487	103.5	+	34	2.29	<0.001**

VC = Vehicle Control, PC = Positive Control, RPE = Relative Plating Efficiency, MTF = Morphological Transformation Frequency, + = normal density/size.
** = $p < 0.01$ (one-sided Fisher's exact test)

6.4.2.4 BioReliance

o-Toluidine HCl was dissolved in DMSO. The concentrations were selected on the basis of the suggested dose range by the VMT, from findings from solubility testing and the DRF test. The results evaluated by BioReliance are shown in Table 26. The MTF values of the test chemical doses ranged from 0.47% to 1.15%. Three test chemical concentrations induced statistically significant increases in MTF compared to the VC ($p < 0.01$).

Table 26: Transformation assay results from BioReliance, testing coded o-toluidine HCl

BioReliance o-toluidine HCl (µg/ml)	Scorable colonies	RPE (%)	Colony density/size	Transformed colonies	MTF (%)	Fisher's exact test *p*-value (one-sided)
VC (DMSO 0.2%)	1330	100.0	+	4	0.30	-
25	1476	111.7	+	7	0.47	0.336
50	1651	124.1	+	12	0.73	0.090
100	1475	111.0	+	17	1.15	0.007**
200	1588	119.4	+	17	1.07	0.011*
300	1540	116.8	++	15	0.97	0.021*
PC	1388	104.4	+	19	1.37	0.002**

VC = Vehicle Control, PC = Positive Control, RPE = Relative Plating Efficiency, MTF = Morphological Transformation Frequency, + = normal density/size, ++ = slightly reduced density/size.
* = $p < 0.05$, ** = $p < 0.01$ (one-sided Fisher's exact test)

6.4.2.5 Concurrent cytotoxicity (Relative Plating Efficiency)

The cytotoxicity curves for Harlan CCR, BioReliance and University of Metz show a similar pattern within the same dose range. In contrast, the results from BASF show a higher, dose-dependent cytotoxicity due to the fact that this laboratory tested at higher concentrations (Figure 13).

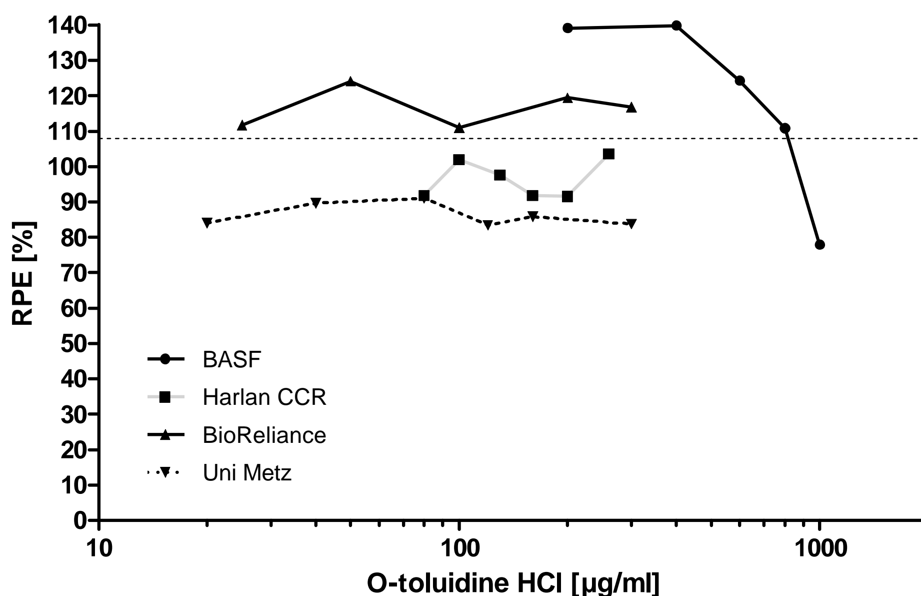


Figure 13: Relative plating efficiency (RPE) for all laboratories, for o-toluidine HCl

6.4.2.6 Morphological transformation frequency

MTF results with o-toluidine HCl are shown in Figure 14. o-Toluidine HCl induced a statistically significant increase in morphological transformation in all laboratories, with a clear dose-dependent effect for Harlan CCR and BASF. University of Metz showed a statistically significant increase in MTF already at very low concentration (20 µg/ml), while for BASF MTF was observed at a higher concentration (600 µg/ml).

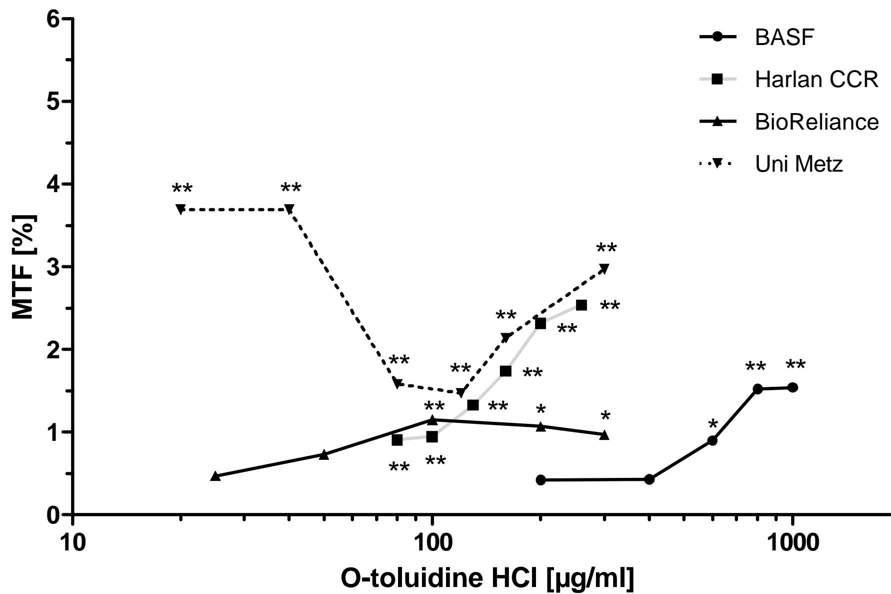


Figure 14: Morphological Transformation Frequency (MTF) shown as a percentage of transformed colonies compared to total colonies, for all laboratories, for o-toluidine HCl
* = $p < 0.05$, ** = $p < 0.01$ (one-sided Fisher's exact test)

6.4.2.7 Acceptance criteria and assessment

The acceptance criteria were fulfilled by all laboratories. o-Toluidine HCl was assessed to be positive by all laboratories (Table 27).

Table 27: Acceptance criteria and assessment of o-toluidine HCl results

o-Toluidine HCl				
Criteria	**Laboratory**			
	Uni Metz	**BASF**	**Harlan CCR**	**BioReliance**
Number of scorable colonies per treatment group • 1000 or <1000 if positive result	**yes** (• 1155)	**yes** (• 1409)	**yes** (• 1321)	**yes** (• 1330)
average of 25-45 colonies per dish or >45 colonies if positive result or <25 colonies if negative result	**yes** (29.9-35.7)	**yes** (35.2-47.6)	**yes** (33.1-37.4)	**yes** (33.3-41.3)
Plating Efficiency of vehicle control >20%	**yes** (21.0%)	**yes** (34.8%)	**yes** (55.6%)	**yes** (27.8%)
Morphological Transformation Frequency of vehicle control <0.6%	**yes** (0.49%)	**yes** (0.26%)	**yes** (0.35%)	**yes** (0.30%)
Number of scorable concentrations • 5	**yes** (6)	**no** (3[#])	**yes** (6)	**yes** (5)
Fisher's test *p*-value of positive control <0.05	**yes** (<0.001)	**yes** (<0.001)	**yes** (<0.001)	**yes** (0.002)
Fulfilment of all assay acceptance criteria	**YES**	**NO[##]**	**YES**	**YES**
Assessment	**+**	**+**	**+**	**+**

[#] results for 200 and 400 µg/ml were not taken into consideration. See section 6.4.2.2 for details.
[##] considered acceptable for the overall study evaluation as there were 3 statistically significant positive concentrations.

6.4.3 Conclusion

Treatment with o-toluidine HCl produced a statistically significant increase in morphologically transformed colonies in all laboratories. As such, o-toluidine HCl was considered to be a positive transforming agent in the SHE pH 7.0 CTA. These results agree with published data (Jones *et al.*, 1988).

6.5 Phthalic anhydride

6.5.1 Dose-range finding test

Figure 15 shows the results of the DRF tests with phthalic anhydride. All laboratories produced very similar cytotoxicity curves.

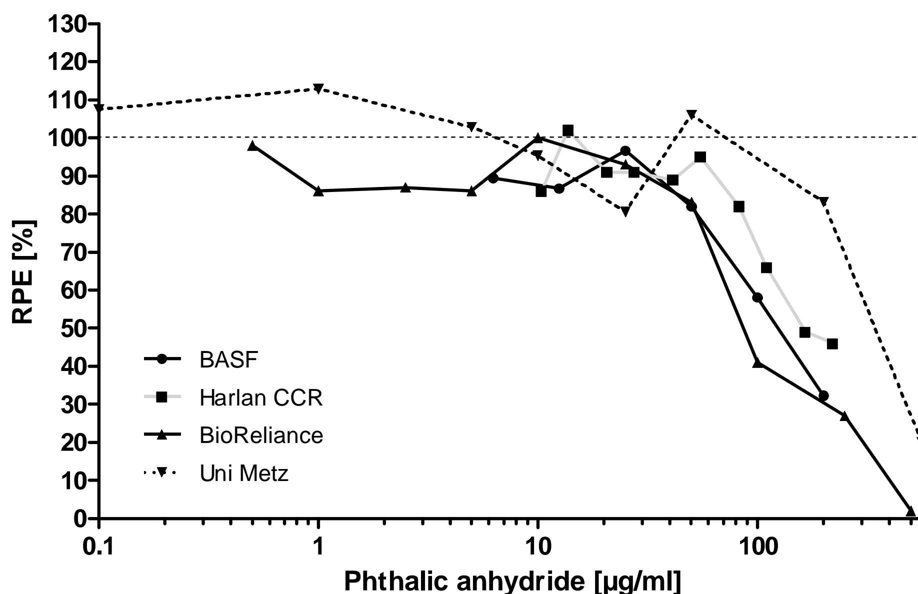


Figure 15: Relative plating efficiency compared to vehicle control in all laboratories testing coded phthalic anhydride, for Dose-Range Finding.

6.5.2 Transformation assay

VCs gave transformation frequencies within the expected range (• 0.6%): 0.56% (University of Metz), 0.26% (BASF), 0.44% (Harlan CCR), and 0.52% (BioReliance). The PC chemical benzo(a)pyrene led to the expected increase in morphologically transformed colonies: 1.60% (University of Metz), 1.43% (BASF), 2.66% (Harlan CCR), and 1.41% (BioReliance). It can be seen from Figure 16 that phthalic anhydride was shown to be cytotoxic in three laboratories (BASF, Harlan CCR and BioReliance). It did not induce statistically significant increase in morphological transformation in most of the laboratories, except at BASF where a statistically significant increase in morphological transformation was observed at a single concentration (120 µg/ml).

6.5.2.1 University of Metz

Phthalic anhydride was dissolved in DMSO. The concentrations were selected on the basis of the DRF test and the chemical solubility and the results evaluated by University of Metz are shown in Table 28. The MTF values of the test chemical doses ranged from 0.34% to 1.11%. None of the test chemical concentrations induced a significant increase in MTF compared to the VC (*p* • 0.05).
The two highest test chemical concentrations were not considered valid because the total number of colonies was below the limit (<1000) with a negative result.

Table 28: Transformation assay results from University of Metz, testing coded phthalic anhydride

Uni Metz Phthalic anhydride (µg/ml)	Scorable colonies	RPE (%)	Colony density/size	Transformed colonies	MTF (%)	Fisher's exact test *p*-value (one-sided)
VC (DMSO 0.2%)	1441	100.0	+	8	0.56	-
0.2	1455	100.2	+	12	0.82	0.258
1	1446	99.8	+	10	0.69	0.409
5	1479	101.9	+	5	0.34	0.272
25	1560	106.9	+	13	0.83	0.244
125	1327	91.2	+	10	0.75	0.339
300	883	60.7	+	6	0.68	0.451
560	631	49.6	+	7	1.11	0.139
PC	1687	116.9	+	27	1.60	0.004**

VC = Vehicle Control, PC = Positive Control, RPE = Relative Plating Efficiency, MTF = Morphological Transformation Frequency, + = normal density/size.
** = $p < 0.01$ (one-sided Fisher's exact test)

6.5.2.2 BASF

Phthalic anhydride was dissolved in DMSO. The concentrations were selected on the basis of the DRF test and the chemical solubility and the results evaluated by BASF are shown in Table 29. The MTF values of the test chemical doses ranged from 0.07% to 0.93%. Treatment with the highest test chemical concentration induced a statistically significant increase in MTF compared to the VC ($p < 0.05$) and the trend test was positive.

Table 29: Transformation assay results from BASF, testing coded phthalic anhydride

BASF Phthalic anhydride (µg/ml)	Scorable colonies	RPE (%)	Colony density/size	Transformed colonies	MTF (%)	Fisher's exact test *p*-value (one-sided)
VC (DMSO 0.2%)	1524	100.0	+	4	0.26	-
40	1513	98.8	+	2	0.13	0.889
60	1412	94.2	+	1	0.07	0.962
80	1487	65.0	+	7	0.47	0.260
100	1417	62.2	+	3	0.21	0.743
120	1606	52.6	++	15	0.93	0.013*
PC	1396	92.0	+	20	1.43	< 0.0005**

VC = Vehicle Control, PC = Positive Control, RPE = Relative Plating Efficiency, MTF = Morphological Transformation Frequency, + = normal density/size, ++ = slightly reduced density/size.
* = $p < 0.05$, ** = $p < 0.01$ (one-sided Fisher's exact test)

6.5.2.3 Harlan CCR

Phthalic anhydride was dissolved in DMSO. The concentrations were selected on the basis of the DRF test and the chemical solubility and the results evaluated by Hralan CCR are shown in Table 30. The MTF values of the test chemical doses ranged from 0.00% to 0.38%. None of the test chemical concentrations induced a significant increase in MTF compared to the VC (*p* • 0.05).

Table 30: Transformation assay results from Harlan CCR, testing coded phthalic anhydride

Harlan CCR Phthalic anhydride (µg/ml)	Scorable colonies	RPE (%)	Colony density/size	Transformed colonies	MTF (%)	Fisher's exact test *p*-value (one-sided)
VC (DMSO 0.2%)	1368	100.0	+	6	0.44	-
27.5	1303	95.4	+	3	0.23	>0.5
41.25	1321	96.6	++	5	0.38	>0.5
55.00	1033	73.7	++	0	0.00	>0.5
82.5	1024	64.9	+++	1	0.10	>0.5
110.0	1036	56.2	+++	0	0.00	>0.5
PC	1430	104.8	+	38	2.66	< 0.0005**

VC = Vehicle Control, PC = Positive Control, RPE = Relative Plating Efficiency, MTF = Morphological Transformation Frequency, + = normal density/size, ++ = slightly reduced density/size, +++ = highly reduced density/size.
** = $p < 0.01$ (one-sided Fisher's exact test)

6.5.2.4 BioReliance

Phthalic anhydride was dissolved in DMSO. The concentrations were selected on the basis of the DRF test and the chemical solubility and the results evaluated by BioReliance are shown in Table 31. The MTF values of the test chemical doses ranged from 0.33% to 0.62%. None of the test chemical concentrations induced a significant increase in MTF compared to the VC (*p* • 0.05).

Table 31: Transformation assay results from BioReliance, testing coded phthalic anhydride

BioReliance Phthalic anhydride (µg/ml)	Scorable colonies	RPE (%)	Colony density/size	Transformed colonies	MTF (%)	Fisher's exact test *p*-value (one-sided)
VC (DMSO 0.2%)	1347	100.0	+	7	0.52	-
2.5	1425	106.3	+	7	0.49	0.646
5.0	1406	104.3	+	5	0.36	0.827
10	1286	95.8	+	8	0.62	0.464
25	1255	93.9	+	7	0.56	0.552
50	1200	89.9	+	7	0.58	0.518
100	1197	44.4	++	4	0.33	0.845
PC	1417	105.6	+	20	1.41	<0.013*

VC = Vehicle Control, PC = Positive Control, RPE = Relative Plating Efficiency, MTF = Morphological Transformation Frequency, + = normal density/size, ++ = slightly reduced density/size.
* = $p < 0.05$ (one-sided Fisher's exact test)

6.5.2.5 Concurrent cytotoxicity (Relative Plating Efficiency)

Cytotoxicity of phthalic anhydride was evaluated by RPE assessment in all laboratories (Figure 16). Phthalic anhydride was shown to be dose-dependently cytotoxic in all laboratories, with the most pronounced effects for BASF, Harlan CCR and BioReliance.

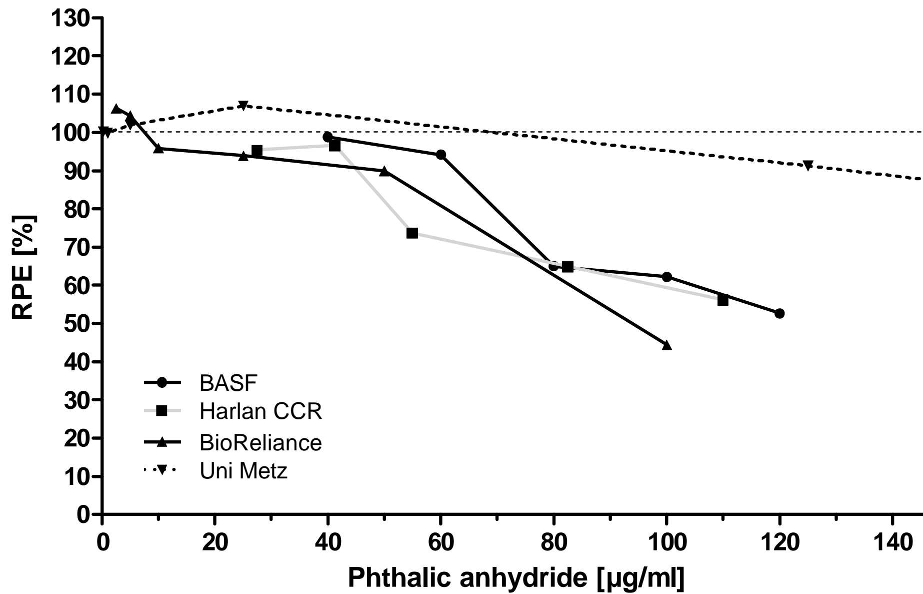


Figure 16: Relative plating efficiency (RPE) compared to vehicle control in all laboratories testing coded phthalic anhydride

6.5.2.6 Morphological transformation frequency

MTF results with phthalic anhydride are shown in Figure 17. The common range tested by all laboratories was from 40 to 100 µg/ml. Phthalic anhydride did not induce a statistically significant increase in morphological transformation in any laboratory, except for BASF, where a statistically significant increase in morphological transformation was observed at the highest concentration only (120 µg/ml).

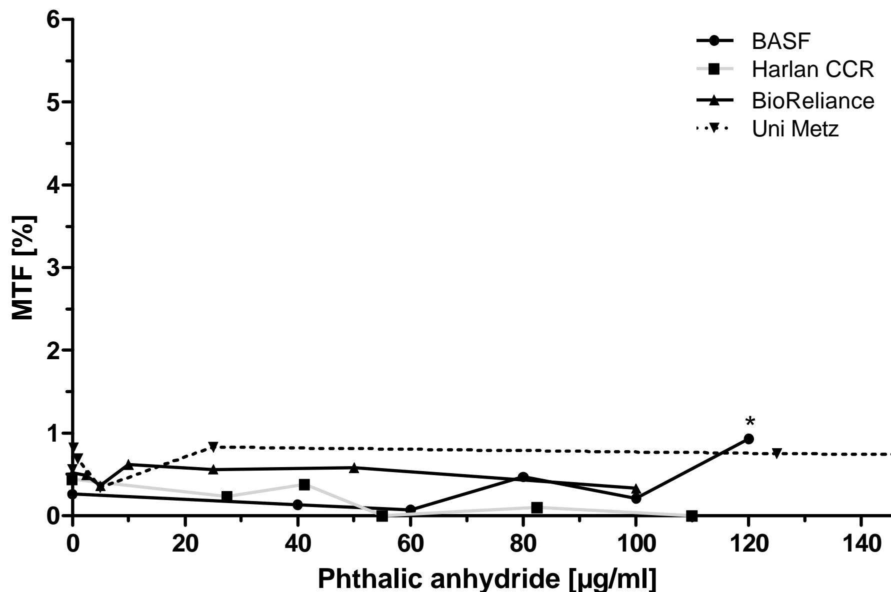


Figure 17: Morphological Transformation Frequency (MTF) shown as a percentage of transformed colonies compared to total colonies for all laboratories testing coded phthalic anhydride

* = $p < 0.05$ (one-sided Fisher's exact test)

6.5.2.7 Acceptance criteria and assessment

The acceptance criteria were fulfilled by all laboratories (Table 32):

Table 32: Acceptance criteria and assessment of phthalic anhydride results

Phthalic anhydride				
Criteria	**Laboratory**			
	Uni Metz	**BASF**	**Harlan CCR**	**BioReliance**
Number of scorable colonies per treatment group • 1000 or <1000 if positive result	**yes** (• 1327)	**yes** (• 1396)	**yes** (• 1024)	**yes** (• 1197)
Average number of 25-45 colonies per dish or >45 colonies if positive result or <25 colonies if negative result	**yes** (33.4-42.7)	**yes** (28.6-40.3)	**yes** (25.2-35.9)	**yes** (29.9-35.8)
Plating Efficiency of vehicle control >20%	**yes** (24.4%)	**yes** (34.8%)	**yes** (52.6%)	**yes** (28.1%)
Morphological Transformation Frequency of vehicle control <0.6%	**yes** (0.56%)	**yes** (0.26%)	**yes** (0.44%)	**yes** (0.52%)
Number of scorable concentrations • 5	**yes** (5#)	**yes** (5)	**yes** (5)	**yes** (6)
Fisher's test *p*-value of positive control <0.05	**yes** (0.004)	**yes** (<0.0005)	**yes** (<0.0005)	**yes** (0.013)
Fulfilment of all assay acceptance criteria	**YES**	**YES**	**YES**	**YES**
Assessment	**-**	**+**	**-**	**-**

results for 300 and 560 µg/ml were not taken into consideration (not valid). See section 6.4.2.1 for details.

6.5.3 Conclusion

Treatment with phthalic anhydride did not produce a statistically significant increase in morphologically transformed colonies in three of the laboratories at any dose tested. In one laboratory a statistically significant increase in morphologically transformed colonies was induced at one concentration only and the trend test was statistically significant, so this chemical was concluded to be positive. As such, under the conditions of the test as performed, phthalic anhydride was considered to be a non-transforming agent in the SHE pH 7.0 CTA in three of the four laboratories. These results agree with published data (Elias *et al.*, 1996).

6.6 Overview on vehicle and positive controls

Vehicle and positive control data for all experiments performed for the between-laboratory reproducibility assessment are summarised in Figure 18. It can be seen that the responses are all within the acceptable limits, *i.e.* below 0.6% for the VCs and above 1.0% for the PCs.

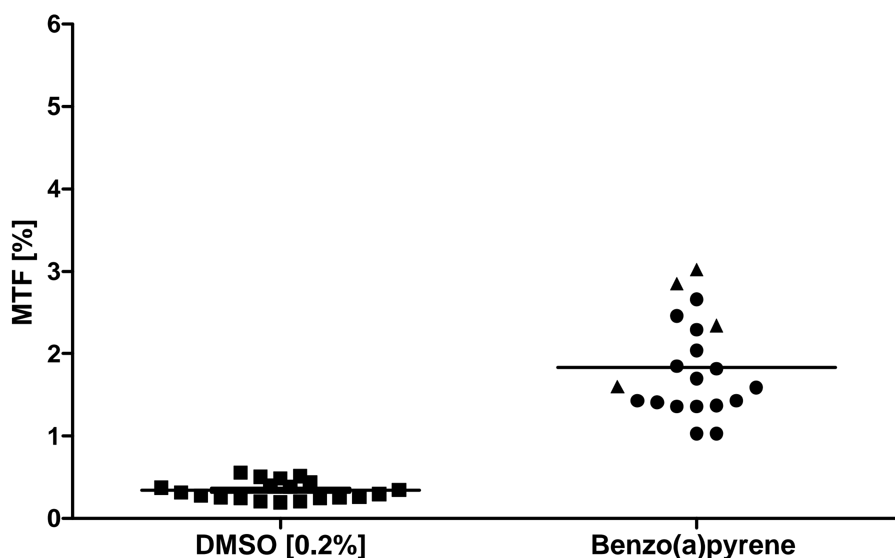


Figure 18: Morphological Transformation Frequency (MTF) of vehicle (•) and positive controls for all cell transformation experiments, in all laboratories. Benzo(a)pyrene was used as positive control at 1 µg/ml by University of Metz (•) and at 5 µg/ml by the other laboratories (•).

6.7 Conclusion of the Validation Management Team on Module 4

The between-laboratory reproducibility was shown to be satisfactory. All chemicals tested as well as vehicle and positive controls gave reproducible results in the four laboratories, but for phthalic anhydride results which were reproducible in three laboratories out of four.

Although it did not impact the overall reproducibility of the results, it can be noted that the cells from University of Metz were in general slightly more sensitive to cytotoxicity and morphological transformation than those from the other laboratories, inducing a statistically significant MTF at lower doses. This could be related to the fact that University of Metz used a different batches of cells and serum than the other laboratories.

Furthermore, for uniformity in testing, all the laboratories were asked by the VMT to dissolve their chemicals in DMSO. In some cases this was not followed and the chemical was dissolved into aqueous medium (DMEM-L). In those cases, a different outcome in results was obtained. In DMSO, the bioavailability of the chemicals towards DNA may have been increased. This emphasizes the importance of vehicle choice in the CTA.

7 Summary of results

An optimised and standardised protocol was initially produced and assessed by University of Metz laboratory and agreed upon by all laboratories before the initiation of the prevalidation study. In addition, training for the participating laboratories and availability of a comprehensive photo catalogue proved to be useful in facilitating the proper conduct and prevalidation of the CTA. These aids helped to ensure consistency in assessing colony morphology and in scoring experimental results, especially in this study where three of the laboratories had no previous experience with this CTA variant. Using the standardised protocol, all laboratories showed good reproducibility within as well as between laboratories. Table 33 summarises the results obtained with each chemical.

Table 33: Summary table of between-laboratory reproducibility results

Chemical	Expected result#	Laboratory			
		BASF	Harlan CCR	BioReliance	Uni Metz
Benzo(a)pyrene	+	+	+	+	+
Anthracene	-	-	-	-	-
2,4-diaminotoluene	+	+	+	+	+
3- Methylcholanthrene	+	+	+	+	+
o-Toluidine HCl	+	+	+	+	+
Phthalic anhydride	-	+	-	-	-

Based on previous results from the literature (see sections 2.4.1.1 and 2.4.1.2)

7.1 Benzo(a)pyrene

The results for benzo(a)pyrene, tested as the PC, showed a good reproducibility within and between laboratories and resulted to be within the acceptable limits requested for the PC in all studies. The experiment using coded benzo(a)pyrene was repeated three times in University of Metz laboratory and performed once by the other laboratories, showing both a good within- and between-laboratory reproducibility.

7.2 Anthracene

The results for anthracene were reproducible between the laboratories and anthracene was shown to be negative in the CTA, which was the expected result.

7.3 2,4-Diaminotoluene

The results for 2,4-diaminotoluene were reproducible between the laboratories and 2,4-diaminotoluene was shown to be positive in the CTA, which was the expected result.

7.4 3-Methylcholanthrene

The results for 3-methylcholanthrene were reproducible between the laboratories and 3-methylcholanthrene was shown to be positive in the CTA, which was the expected result.

7.5 o-Toluidine HCl

The results for o-toluidine HCl were reproducible between the laboratories and o-toluidine HCl was shown to be positive in the CTA, which was the expected result.

7.6 Phthalic anhydride

The results for phthalic anhydride were reproducible in three laboratories out of four. These laboratories showed that phthalic anhydride was negative in the CTA, which was the expected result, whereas BASF observed a significant increase in MTF at a single concentration only, with a positive trend test.

8 Overall conclusion by the Validation Management Team

The aim of the study was to prevalidate the SHE CTA at pH 7.0, in a formal inter-laboratory study, following the modular approach (Hartung *et al.*, 2004) and concentrating on the modules 1-4: test definition, within-laboratory reproducibility, transferability and between-laboratory reproducibility. Table 34 summarises the conclusion by the VMT on the assessment of the SHE pH 7.0 CTA.

Table 34: Conclusions of the Validation Management Team for the different modules

Module		Summary & Conclusion	
Module 1	**Test definition**	- Clear definitions of the scientific basis - description of the endpoint induced by genotoxic and non-genotoxic mechanisms - improved protocol available	**yes**
Module 2	**Within-laboratory reproducibility**	The within-laboratory reproducibility was shown to be satisfactory in all laboratories for: - the vehicle control - the positive control - the test chemicals (tested in a single laboratory)	**yes**
Module 3	**Transferability**	- The test method is transferrable between laboratories - Basic cell culture experience is needed - Training in the conduct and scoring of the assay is important - Photo catalogue produced as a useful aid for scoring	**yes**
Module 4	**Between-laboratory reproducibility**	The between-laboratory reproducibility was shown to be satisfactory for: - the vehicle control - the positive control - the test chemicals	**yes**

The VMT concluded that in keeping with the objectives of this CTA effort, the SHE pH 7.0 CTA had been prevalidated in accordance with modules 1-4 (Hartung *et al.*, 2004). It has been demonstrated that a standardised protocol is available that should be the basis for future use. This protocol and the assay system itself are transferable between laboratories, and reproducible within- and between-laboratories.

This conclusion is substantiated by the existing body of knowledge related to this assay. In particular, by 1) the reproducibility evaluations of similar protocols as reported in the literature (Isfort *et al.*, 1996c) and, 2) the overall evaluation of the SHE data contained in the OECD DRP, which reported consistent results for 87.7% (57/65) of chemicals which had been tested in more than one laboratory (OECD, 2007). Moreover, the VMT concluded that with the appropriate training and the use of the photo catalogue, colony scoring was not problematic despite the concerns raised in the past.

In addition, the data produced add to the understanding of the predictive capacity (module 5) of the CTA, which was previously addressed by the OECD DRP evaluation (OECD, 2007).

The VMT supports the conclusions of the OECD DRP and the generation of an OECD SHE cell transformation test guideline.

9 Recommendations

Taking into account clarifications and minor modifications introduced by the VMT and the participating laboratories, the VMT agreed that a standardised, transferable and reproducible protocol has been established. This protocol will be published by ECVAM and used in the future to produce new data and an OECD test guideline. Based upon the experience gained in this effort, points that need to be taken into consideration in the future conduct of the assay and generation of the OECD guideline include the following:

- Considering the limited differences between the protocols for the SHE CTA at pH 6.7 and pH 7.0, it is recommended that both CTAs be incorporated into a single protocol since they only differ by the pH used to culture the cells and the morphology of the transformed colonies.

- Since there is a certain degree of subjectivity associated with the identification of transformed colonies in the SHE CTA and correct scoring of transformed colonies is critical, training is necessary to ensure scoring which is as consistent and objective as possible. It needs to be noted that the SHE protocol at pH 7.0 produces colonies with slightly different morphologies from those produced with the protocol at pH 6.7. Nevertheless, the successful assessment of the between-laboratory reproducibility demonstrated that if the laboratories are well trained, the manual scoring of colonies and the potential subjectivity in identifying transformed colonies are not problematic issues. It is therefore recommended that appropriate training and a photo catalogue for this specific pH protocol variant be made available to laboratories conducting the SHE CTA. It is intended that such a photo catalogue will be published by ECVAM in the near future for that purpose.

- It is important to note that cells for this assay need to be isolated at pH 7.0.

- For results that do not meet the criteria for a clear positive or a clear negative call (inconclusive results) the experiment should be repeated, as is normal practice in assays in general.

- In case of at least 2 statistically significant concentrations, a positive call could be concluded regardless of the number of scorable concentrations *(i.e.* those that fulfil the assay acceptance criteria) as opposed to a specific requirement for a set number of concentrations (see also point below).

- Based upon the experience of the participating laboratories and the results obtained, the VMT recommends that one of the acceptance criteria be revised as follows: "At least four valid test chemical concentrations should be available to evaluate a negative call", instead of a requirement for five concentrations.

- It is recommended that clarifications on terminology be made as required (for instance, negative control should be understood as untreated control) and will be taken into account in the published protocol and for the drafting of the OECD guidelines.

10 Current contact details of the people involved in the prevalidation Study

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12 Annexes

12.1 Chemicals selected for the prevalidation of SHE pH 7.0 CTA

Chemicals were selected based on the genotoxicity and carcinogenicity data compiled by the OECD DRP31 (2004; 2007) and Kirkland *et al.* (2005).The *in vitro* genotoxicity, *in vivo* genotoxicity and carcinogenicity characterisation of the selected compounds is reported in Table 35.

Table 35: Genotoxicity and carcinogenicity data on the chemicals selected for the SHE pH 7.0 CTA prevalidation study

Chemical	CAS number	Genotoxic profile *in vitro*				Genotoxic *in vivo*	IARC class	*in vivo* carcinogenicity
		Ames	MLA	MNT	CA			
Benzo(a)pyrene	50-32-8	+	+	+	+	+ (gene mutation, MN)	1	+
Anthracene	120-12-7	+/-	+	nd	-	i	3	-
2,4-Diaminotoluene	95-80-7	+	+	nd	+	+ (UDS, transgenic mutant, comet) - (MN)	2B	+
3-Methylcholanthrene	56-49-5	+	+	+	+	i	nd	+ *
o-Toluidine HCl	636-21-5	+/-	+/-	+/-	+/-	+/-	2A	+
Phthalic anhydride	85-44-9	-	+	nd	+	- (gene mutation)	nd	- **

* source: Gold and Zeiger (1997); ** source: NTP database
+: positive; -: negative; +/-: diverging results inside a database; nd: not determined; i: inconclusive result;
MLA: Mouse Lymphoma Assay; MNT: Micronucleus Test; CA: Chromosome Aberration; UDS: unscheduled DNA synthesis; MN: micronucleus.

12.2 SHE pH 7.0 Cell Transformation assay protocol

SOP

PREVALIDATION STUDY ON
SYRIAN HAMSTER EMBRYO (SHE) *IN VITRO*
CELL TRANSFORMATION ASSAY

SHE ASSAY pH 7.0

Written by LIEBE CNRS UMR 7146 - UPV Metz

1. CONTENTS

1.	**CONTENTS**	**2**
2.	**INTRODUCTION**	**4**
3.	**PRINCIPLE**	**5**
4.	**MATERIALS AND METHODS**	**6**
4.1.	Preparation of SHE cells	6
4.2.	Test chemical: provision and handling	6
4.3.	Culture media and reagents for testing	6
4.4.	Preparation and cryopreservation of SHE cell stocks	7
4.5.	Irradiation of SHE cells	8
4.6.	Equipment	9
5.	**EXPERIMENTAL DESIGN**	**10**
5.1.	Time schedule	10
5.2.	Preparation of test cultures	10
5.3.	Preliminary Cytotoxicity Assay / Dose range finding study	11
5.4.	Dose selection for the main experiment	11
5.5.	Controls	12
5.6.	Cell Transformation Assay	12
5.7.	Determination of Solubility, pH and Osmolality	12
6.	**EVALUATION**	**13**
6.1.	Morphological Cell Transformation	13
6.2.	Evaluation criteria	13
6.3.	Cytotoxicity	13
6.4.	Statistics	14
6.5.	Acceptance Criteria and historical controls	14
6.6.	Assessment criteria	14
7.	**DOCUMENTATION AND DEVIATION**	**15**
8.	**REFERENCES**	**15**
9.	**ANNEX I**	**16**
9.1.	Test Chemical Receipt Page	16

10. ANNEX II **17**
10.1. Test Reporting Template for Chemical Dilution 17
11. ANNEX III **18**
11.1. Test Reporting Template for Pre-validation of SHE assay 18
12. ANNEX IV **19**
12.1. Template for preparation of feeder cells 19
12.2. Template for preparation of target cells 20
13. ANNEX V **21**
13.1. Template for Test Substance Preparation 21
13.2. Template for Seeding and Treatment 22
13.3. Template for Treatment Conditions 23
14. ANNEX VI **24**
14.1. Template for Preliminary Cytotoxicity Assay 24
14.2. Template for Morphological Transformation – Raw data 25
14.3. Template for Morphological Transformation – Table Results 26

2. INTRODUCTION

Syrian Hamster Embryo (SHE) cells have been used to study transformation *in vitro* since Berwald and Sachs (1963) confirmed the key observation by Earle (1943) that morphological changes in cell cultures were associated with oncogenicity of these cells *in vivo*. These authors demonstrated oncogenicity of SHE cells which presented a transformed phenotype after exposure to chemical carcinogens *in vitro*.

SHE cells are primary and normal diploid cells, which derive from mid-gestation embryos (13 - 13.5 days gestation). The cells are genetically stable, metabolically competent and have a finite life span. The cell population isolated from embryos of pregnant Syrian Hamster comprises of multiple cell types and cells at various stages in the differentiation process and hence provides a broad spectrum of cellular targets for neoplastic transformation. SHE cells show a high proliferation rate, good plating efficiency (20 - 40 %) and a low spontaneous transformation frequency. Exposure to carcinogenic agents results in an increase in the percentage of morphologically transformed (MT) colonies compared to controls. MT colonies are characterized by a random growth pattern, which expresses a loss of growth inhibition and cell-cell orientation at confluency.

The Syrian Hamster Embryo *in vitro* cell transformation assay provides a system to detect carcinogenic chemicals that act via genotoxic and non-genotoxic mechanisms. The assay is proposed as an alternative to long term rodent experiments for carcinogenicity.

Study of morphological transformation *in vitro* in SHE cells is in agreement with the OECD draft proposal (*In vitro* Syrian Hamster Embryo (SHE) Cell Transformation Assay, 1996). In the cell transformation assay (CTA), SHE cells are used to estimate cytotoxicity and morphological transformation. The endpoint of cytotoxicity is the formation of colonies (Plating efficiency, PE) and the endpoint of the carcinogenic potential is the presence of morphologically transformed colonies. The treatment time is 7 days and duration of the test is 9 days in total.

This protocol describes the procedure of the SHE assay carried out at pH 7.0 for phase I of the prevalidation study. The test should meet the acceptance criteria on reproducibility as defined by ECVAM principles for test validity (Hartung *et al.*, 2004).

The test reproducibility will be assessed using benzo[a]pyrene (B[a]P) which will be tested as a test substance in three independent assays. B[a]P tested had been provided as chemical Y by ECVAM in November 2005.Assays will be conducted on batch of SHE cells isolated in complete growth medium at pH 7.0 in February 2006 and checked for their performances as cell reagents in CTA in May 2006. Number of target cells is 150 cells/plate, number of feeder cells is 50 000 cells/plate.

3. PRINCIPLE

SHE cells are obtained from primary culture of individual Syrian hamster embryos at 13 days of gestation. After enzyme tissue digestion, cells are collected and stored in liquid nitrogen. One part of cryopreserved SHE cells will be used as feeder cells, the other part as target cells. These ‘feeder cells’ will be X-ray irradiated to be no longer capable of replication and seeded as nutrient base and support for metabolic activity.

The principle of the test consists in seeding target cells at clonal density onto a feeder layer of X-ray - irradiated cells. Twenty-four hours after seeding feeder cells, the target cells are seeded onto the feeder layer at a density appropriate to obtain 25-45 colonies per plate (60 mm diameter) and treated 24 hours later. After 7 days necessary for clonal expansion, cells are washed, fixed and stained with Giemsa. Dishes are coded and colonies are scored for their morphological phenotype under stereomicroscope.

Cytotoxicity is evaluated by inhibition of cloning efficiency. The number of MT colonies reported to the total number of scorable colonies is calculated for each concentration tested. A statistically higher percentage of MT colonies at two concentration levels compared to control vehicle will conclude to positivity.

The test medium is the Dulbecco’s modified Eagle’s medium (DMEM) containing 1 g/l of glucose, without phenol red, supplemented with fetal calf serum (15%) and pH of the test medium is 7.0.

The test performances of the protocol of SHE cell transformation assay at pH 7.0 will be assessed on three independent assays, conducted using:

- B[a]P as test substance (chemical Y provided by ECVAM in November 2005)
- SHE target cells isolated at pH 7.0 (batch T1) : 150 cells/plate, seeded on a feeder layer of 50 000 cells/plate
- DMEM (low glucose, without phenol red, pH 7.0) added with
- 15% fetal bovine serum (Hyclone Perbio AQL 25 247)

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4. MATERIALS AND METHODS

4.1. Preparation of SHE cells

For this prevalidation study phase I, SHE cells are prepared from embryos as described in section 4.4. The cell batch selected is No T1. SHE cells were isolated from one embryo in February 2006. The SHE cells of batch T1 were checked for their performances - spontaneous transformation rate, plating efficiency and its ability to form morphologically transformed colonies after exposure to B[a]P (7 days at 1 µg/ml) - in May 2006.

4.2. Test chemical: provision and handling

Benzo[a]pyrene has been selected as the test substance for the assays/Phase 1. The test chemical is the coded chemical Y provided by ECVAM in November 2005.

Appropriate routine safety procedures will be followed in handling the test chemicals, unless otherwise specified. Test facility personnel has been instructed to treat B[a]P and all other coded test chemicals as very hazardous and potentially carcinogenic and to dispose laboratory wastes as toxic wastes.

4.3. Culture media and reagents for testing

- **Culture media and reagents**
- **DMEM** : Dulbecco's Modified Eagle's Medium containing 1 g/l of glucose, 4 mM of glutamine and 110 mg/l of sodium pyruvate, without phenol red (Invitrogen, Gibco 31600). Lyophilized.
- **FBS**: Fetal Bovine Serum (Perbio, Hyclone SH3-0070. Lot AQL 25 247). FBS is inactivated at 56°C for 30 minutes.
- **PBS-CMF**: calcium and magnesium-free phosphate buffer saline.
- **Trypsin** 2.5% (×10) liquid (Invitrogen, Gibco No.15090-46)
- **Sodium bicarbonate $NaHCO_3$** (Sigma, No S-5761).

- **Dissociation solution**

Dispase (0.5 U) (Roche No. 165859) used at concentration 1.2 U/ml in PBS-CMF.

The dissociation solution for cell isolation is prepared and used freshly in each case.

- **CGM : Complete Growth Medium**

The lyophilized DMEM medium (DMEM) is reconstituted with ultrapure water, then adjusted to pH 7.0 with 1.5 g/l of $NaHCO_3$ and sterilized by membrane filtration (0.1 µm porosity). The culture medium is stored at 4° C during a period not exceeding 2 weeks. The complete culture medium is prepared with addition of 15% FBS.

The pH of the culture medium is checked so as to be 7.0 after incubation at 37.0 ± 0.1°C with 10.0 ± 0.5% CO_2 in a humidified incubator for at least 4 hours.

- **Detachment solution**

The cell detachment solution consists of calcium/magnesium-free phosphate buffer saline (PBS-CMF) containing 0.25% trypsin .

§ **Cryopreservation Medium**

DMEM pH 7.0, added with :
- 10% FBS and
- 10% DMSO (Sigma D-8779)

§ **Cell isolation medium (CIM)**

The cell isolation medium is constituted with DMEM (1.5 g/l of $NaHCO_3$) added with 15% FBS and 1% antibiotics (penicillin 5000 U/ml ; streptomycin 5000 µg/ml)

§ **Wash solution for cell isolation**

The wash solution is PBS-CMF with 1% antibiotics (5000 U/ml ; streptomycin 5000 µg/ml)

§ **Stain Solutions**

Trypan Blue Stain (0.5% in PBS) (ICN Biomedicals, No. 195532).
Giemsa Stain (10% Giemsa in water) (Sigma, No. 48900).

4.4. Preparation and cryopreservation of SHE cell stocks

Sacrifice of hamster to obtain embryos

- Sacrifice a pregnant hamster at 13 days of gestation by guillotine.
- Swab ventral surface of hamster with betadin.
- Incise and retract abdominal skin and open peritoneal cavity in sterile conditions.
- Remove both uterine horns containing embryos and place them in sterile ø100 mm culture dishes containing wash solution.

Dissociation of embryonic tissue

- Remove embryos from uterus and transfer to sterile ø100 mm culture dishes containing wash solution. After two washings in wash solution, embryos are transferred in culture dishes containing cell isolation medium (CIM). The differentiated organs (eyes, heart and viscera) are removed from each embryo.
- Cut tissue into thin pieces with sterile curved scissors.
- Transfer minced tissue to erlenmeyers (one erlenmeyer for one embryo) containing a magnetic stir bar on a stir plate and rinse the minced tissue with wash solution at slow stirring speed to remove as many blood cells as possible at 37.0 ± 0.1°C. Allow tissue to settle then pipette off and discard wash solution.
- Add dissociation solution to the flask and gently stir tissue for 5 minutes at 37.0 ± 0.1°C.
- Allow tissue to settle and pipette off dissociation solution and discard.
- Add dissociation solution to the flask and gently stir tissue for 10 minutes at 37.0 ± 0.1°C.
- Put FBS to a series of sterile plastic centrifuge tubes.
- After 10 minutes with dissociation solution, allow tissue to settle and carefully pipette off supernatant (dissociation solution and cells) and after filter through the sterile gauze into the prepared centrifuge tubes.
- Add fresh dissociation solution to the tissue and gently stir for 10 minutes. Collect the solution and cells as describe above.

- Repeat dissociation 2-4 times.
- Centrifuge the cell suspension at 250×*g* for 10 minutes at 4°C. Discard the supernatant, resuspend it and pool cells in CIM.
- Count the viable cells by Trypan Blue Dye exclusion and seed 2×10^6 viable cells/culture dish. Place culture dishes in incubator (37.0 ± 0.1°C and 10.0 ± 0.5% CO_2).
- 24 hours after seeding, cryopreserve cells when at 60-80% confluency.
- If 60-80% confluency is not reached within 24h, remove growth medium and refeed culture dishes with CIM. Return culture dishes to incubation and collect cells when appropriate confluency is reached.

Cryopreservation of the cells

- When cells are 60-80% confluent, remove growth medium from the culture dishes and rinse cell layers with PBS. Detach cells with detachment solution.
- Add CIM to culture dishes and pool cells in centrifuge tubes.
- Collect cells by centrifugation at 250×*g* for 10 minutes.
- Discard supernatant and add CGM.
- Count the viable cells by Trypan Blue Dye exclusion. Centrifuge at 250×*g* for 10 minutes and resuspend the pellet with the cryopreservation medium at 1×10^6 cells/ml.
- Distribute 1.5 ml of cell suspension into storage vials (1.5×10^6 cells/vials).
- Step freeze cells by placing vials into ice for 30 minutes, -20°C for 4 hours and -80°C for one night, prior to transfer and store under liquid nitrogen.

Checking of the SHE cells

Before use, each new cell batch is checked for:

- Spontaneous transformation rate
- Plating efficiency (= colony forming ability)
- Morphological transformation with the standard carcinogen B[a]P

4.5. Irradiation of SHE cells

- Reconstitute cryopreserved SHE cells by gently thawing (37°C) frozen vials and transferring cells into centrifuge tubes with fresh CGM. Centrifuge at 250×*g* for 10 minutes to eliminate DMSO. Resuspend the pellet in 10 ml fresh CGM and transfer 1.5×10^6 cells in each ø100 mm culture dishes.
- Grow cells in incubator for 2-3 days to achieve 50-90% confluency.
- On the day of irradiation, rinse each plate with 10 ml of PBS and cover cells briefly with 1 ml of detachment solution. Observe detachment with a phase microscope.
- Immediately after detachment, resuspend cells in 460 ml CGM in a 175 cm² growth flask on wet ice.
- Expose cells to X-ray irradiation (5000 rads) so that they are still viable, yet no longer capable of replication.
- Transfer cells to centrifuge tubes and centrifuge in a refrigerate unit to form a pellet. Remove supernatant medium and resuspend pellet in 50 ml of CGM on wet ice.
- Count the viable cells using the Trypan Blue dye exclusion test.
- Distribute 1.5 ml of cells suspension into storage vials (5×10^6 cells/vials) on wet ice, then at 4°C for 30 minutes, -20°C for 4 hours and -80°C for one night, prior to transfer and storage under liquid nitrogen.

4.6. Equipment

- Laminar flow hood (biohazard type and restricted to cell culture assays)
- Cell culture incubators (37.0 ± 0.1°C; 10.0 ±0.5% CO_2; 90 ± 5% humidity)
- Low-speed centrifuge
- Water bath (37°C)
- Inverse phase microscope
- Micropipettes
- Computer
- Refrigerator (4°C)
- Freezers (-20°C)
- Containers for storage in liquid nitrogen
- Autoclave (for instruments and for bio-hazardous waste materials)
- Balance
- pH meter
- Osmometer
- Cell counting system
- General cell culture laboratory equipment (*e.g.* glassware, filtration systems, cell culture plastic-ware, etc...)
- X-ray machine (Philips SL25)

All equipment maintenance and calibration shall be routinely performed in GLP spirit.

5. EXPERIMENTAL DESIGN

5.1. Time schedule

Day 0	1	2	9
↑	↑	↑	↓
Feeder cells (2 ml)	Target cells (2 ml)	Treatment with test substance (4 ml)	Fixing Staining

5.2. Preparation of test cultures

5.2.1. Feeder cells

On day 0 (feeder cells day), the cryopreserved irradiated SHE cells are thawed (37°C) and transferred into a centrifuge tube with 10 ml of fresh complete growth medium. Centrifuge at 250×*g* for 10 minutes to eliminate DMSO and resuspend the pellet in 10 ml fresh CGM.
Count the viable cells using the Trypan Blue dye exclusion test and adjust the cell concentration to 25000 cells/ml in CGM and add 2 ml into each ø60 mm culture dish.
The culture dishes are incubated at 37.0 ± 0.1°C and 10.0 ± 0.5% CO_2 in humidified air for 24 hours before adding target cells.

For each test, at least 5 dishes filled with feeder cells only will be used concurrently to check the inability to replicate and to form colonies. No colony must form in these dishes.

5.2.2. Target cells

Cryopreserved SHE cells are thawed and seeded for growth in culture flasks. After an incubation period of 24 hours, the target cells will be detached, counted with a hemocytometer and adjusted with the complete growth medium to a concentration allowing us to obtain approximately 25 - 45 colonies/dish at the end of the test. Two ml of the target cell suspension will be added into each culture dish containing 50000 feeder cells. Dishes will be incubated in a humidified incubator (37.0 ± 0.1°C; 10.0 ± 0.5% CO_2) for 24 hours prior to treatment.

In case of cytotoxicity at certain dose groups, the target cell number is adjusted so as to obtain the required number of colonies at the end of the test. The target cell number is determined on the basis of the results of the cytotoxicity assay.

5.2.3. Preparation of test solutions and treatment of cultures

The solutions of the test substance will be prepared 24 hours after the seeding of target cells, on the day of treatment. Each dosing solution will be prepared at a concentration corresponding to 2x the final concentration expected and 4 ml of each dosing solution will be transferred to culture dishes (ø60 mm) already containing 4 ml of complete medium with feeder and target cells (final volume 8 ml). The cultures will be incubated for 7 days in a humidified incubator (37.0 ± 0.1°C; 10.0 ± 0.5% CO_2).

A series of solutions of the test substance (500x the final concentration) in the appropriate vehicle will be prepared so as to obtain the final desired concentration x in the test medium, as follows:

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

*For non hydrosoluble test substances, concentrated solutions (500x) will be prepared in DMSO

5.3. Preliminary Cytotoxicity Assay / Dose range finding study

The maximum dose of the test substance will be determined taking into account the solubility and any relevant cytotoxicity information available for the test substance. A range of at least 10 concentrations to achieve a wide toxicity range will be tested in parallel to the vehicle. Ten dishes will be seeded per concentration tested. At this stage, the number of target cells seeded is the same in all dose groups. The conditions of testing (test medium, incubation conditions and time) are the ones described for the main experiment for cell transformation (see section 5.6).

The highest dose level tested for soluble test substance will be 5 mg/ml or 10 mM. Test substance solutions will be prepared fresh on the day of treatment.

The cultures will be incubated for a period of 7 days to allow colony development. The relative cytotoxicity of each treatment group will be measured by the reduction in plating efficiency and/or colony density and size of the treated SHE cells compared to the vehicle control group.

5.4. Dose selection for the main experiment

The relative cytotoxicity of each treatment group will be assessed from the dose range-finding study. The results will be evaluated by the study Director for selecting doses to be used in the SHE cell transformation assay. Unless there is evidence of MT, definitive assay doses should include, if possible:

- A high dose causing an approximate 50% decrease in relative plating efficiency and/or ≥ 50% reduction in relative colony density/size (by visual appearance).
- At least one dose which has no effect on plating efficiency.
- 3 or 4 intermediary doses

If the test substance is essentially non-toxic, then at least five concentrations will be selected up to a maximum of 5 mg/ml or 10 mM. For non-toxic and insoluble test substances, the highest dose level tested will be within 2-times the visible solubility limit in complete medium. For toxic and insoluble test substance, the highest dose level tested should cause an approximate 50% decrease in relative plating efficiency or relative colony density, regardless of the number of insoluble dose levels.

5.5. Controls

5.5.1. Vehicle control

In case the test substance is not hydrosoluble, the vehicle control selected will be DMSO used at a concentration that will not exceed 0.2%. The final concentration of DMSO will be the same in all vehicle control and treated dishes: 0.2%.

5.5.2. Positive control

Benzo[a]pyrene (B[a]P) will be tested at a concentration of 1.0 µg/ml to demonstrate the sensitivity of the assay. B[a]P will be dissolved in DMSO.

5.6. Cell Transformation Assay

A sufficient number of target cells (around 150 cells/dish) to produce an average of 25 - 45 colonies will be dispensed in 2 ml of complete medium per ø60 mm culture dish, each of which was seeded approximately 24 hours earlier with 5×10^4 feeder cells in 2 ml of complete medium. In case of cytotoxicity, the cell number should be adjusted: *e.g.* a reduction of approximately 30% needs a 1.5x adjustment; a reduction of approximately 50% needs a 2x adjustment.

The transformation assay may be a single trial that results in at least 1000 colonies per treatment group (total of 40 dishes) to establish a negative result. The assay should include at least 5 scorable concentrations of the test compound and the appropriate vehicle and positive control[1].

The dose cell cultures will be incubated for a period of 7 days in a humidified incubator (37.0 ± 0.1°C; 10.0 ± 0.5% CO_2) following treatment initiation. The culture dishes will be labelled with a code/assay number, trial number and dose level.

After the incubation period of 7 days, the medium is aspirated off and the dishes are rinsed with 3-4 ml PBS-CMF. After removal of PBS, cells are covered with 5 ml of ethanol for 10 minutes. Ethanol is then removed and plates stained for 20 minutes with 3-5 ml of Giemsa stain (10% in pure water). Stain is poured off and plates rinsed under tap water before cells are air-dried.

5.7. Determination of Solubility, pH and Osmolality

The solubility of the test article in the vehicle and in the test culture (medium) will be observed and documented.

The pH of the test article dosing solutions will be checked after at least four hours of undisturbed incubation in humidified atmosphere at 37.0 ± 0.1°C and 10.0 ± 0.5 % CO_2. Prior to performing the preliminary cytotoxicity assay, the test article will be dissolved in an appropriate solvent and diluted in complete medium at a concentration equal or greater than the highest concentration to be tested. The pH will be determined using a standard pH meter both at the time of preparation and after at least 4 hours of incubation.

The osmolality will be determined using a suitable osmometer and may be measured prior to or at the time of performing the preliminary cytotoxicity determination or the main experiment.

[1] In this Phase I study, as the test substance is B[a]P (compound Y) no other positive control will be performed.

6. EVALUATION

6.1. Morphological Cell Transformation

The stained colonies are blindly scored under stereomicroscope for plating efficiency and morphological transformation.
Morphologically-transformed colonies are characterized by a multi-layered, criss-cross pattern of growth throughout the colony and piling up of cells.
Sparse colonies are not scored for MT, however included in the total number of colonies for plating efficiency determination. If the colony contains less than 50 cells, it is not counted and recorded. Colonies at the edge of the plates can be scored for MT if clearly morphologically transformed.
Generally, for each test group ≥ 1000 colonies will be evaluated for morphological cell transformation.
A differentiation will be made between normal (non-transformed) colonies and transformed colonies.

6.2. Evaluation criteria

Morphologically transformed colonies contain cells in an extensive, random-oriented, three-dimensional growth pattern with cell stacking and criss-crossing both at the colony centre and on the perimeter. Individual cells within the colony are more basophilic relative to their normal counterparts and have a decreased cytoplasm-to-nucleus ratio.

The morphological transformation frequency (MTF) will be calculated for each test group as follows:

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

6.3. Cytotoxicity

6.3.1. Relative Plating Efficiency (RPE)

§ Relative plating efficiency

The average number of colonies per dish, the plating efficiency and the relative plating efficiency (RPE) will be determined for each test group.

The plating efficiency and the relative plating efficiency will be calculated as follows:

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

The relative plating efficiency :

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

§ Colony size and density

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

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

6.4. Statistics

The data of one or several trials are pooled for each treatment group. Results are analysed using the one-sided Fisher's exact test (Armitage, 1955) to determine if an increase in morphological transformation occurred compared to vehicle control.
The Cochran-Armitage trend test for a positive dose-related response is performed when only one chemical concentration shows a statistically significant response.

6.5. Acceptance Criteria and historical controls

- 1000 colonies per treatment group should be available for morphological transformation (less than 1000 colonies is acceptable in case of significant increase in morphological transformation rate). However the average number of colonies per plate should not be less than 25.
- An average of 25-45 colonies per dish for each treatment group (a colony number beyond these limits is acceptable in the case of negative results with < 25 colonies or positive results with > 45 colonies per dish).
- Cloning efficiency of the negative/vehicle control should be ≥ 20%.
- No colony formation should be observed in the feeder cell control dishes. Feeder cells must be visible in the chemical treatment groups except if they are affected selectively by the compound.
- Transformation frequency in the negative controls (untreated and vehicle) within historical controls.
- Increase in morphological transformation frequency of the positive control (this criterion can be discussed and should not be required in case of significant positivity of the test substance).
- The positive control substance must lead to a statistically increase of morphological cell transformation.
- There should be at least 5 scorable concentrations.

Historical controls are :

- 0.28 ± 0.22 with negative control and 0.36 ± 0.31 with DMSO,
- 1.93 ± 0.78 with 1µg/ml B[a]P

6.6. Assessment criteria

A test chemical is declared **positive** if it causes a significant increase in morphological transformation frequency either in two successive concentrations compared to the vehicle control or if one concentration shows a statistically significant increase and the trend test is significant.
Otherwise, a test chemical is declared **negative**, *i.e.* no significant increase in morphological transformation or only at one dose without a significant trend test.

7. DOCUMENTATION AND DEVIATION

The prevalidation study will be conducted in the spirit of GLP.
All the data will be recorded in the study workbook and results registered in labelled files which will be provided in the draft report.
The culture dishes of all the experiments will be archived until the end of the prevalidation study.

Deviations from the Statement of work or these SOP will be documented in the study workbook and countersigned by the study director. They will be indicated in the draft report.

8. REFERENCES

Armitage P. (1955) Test for linear trend in proportions and frequencies. Biometrics 11, 375-386.

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

Earle W.R. (1943) Production of malignancy " in vitro ". IV. The mouse fibroblast cultures and changes seen in the living cells. *JNCI*, 4, 165-212.

Hartung T., Bremer S., Casati S., Coecke S., Corvi R., Fortaner S.; Gribaldo L., Halder M., Hoffmann S.; Roi A.J., Prieto P.; Sabbioni E., Scott L., Worth A., Zuang V. (2004) A modular approach to the ECVAM principles of test validity. *ATLA*, *32*, 467-472

9. ANNEX I

9.1. Test Chemical Receipt Page

Test Facility Test Chemical Identification Number	Test Chemical Physical Description	Storage Conditions	Test Chemical Receipt Date	Test Chemical Received By	Comments

10. ANNEX II

10.1. Test Reporting Template for Chemical Dilution

Testing Facility___________________

Test Chemical Code__________

Physical Description_____________________________Liquid Density_________

Date______________

<table>
<tr><th>Solvent</th><th>Amount of Test Chemical</th><th>Total Volume</th><th>Final Concentration</th><th>Comments</th></tr>
<tr><td rowspan="3">Routine Culture Medium</td><td rowspan="3"></td><td></td><td></td><td></td></tr>
<tr><td></td><td></td><td></td></tr>
<tr><td></td><td></td><td></td></tr>
<tr><td>DMSO</td><td></td><td></td><td></td><td></td></tr>
</table>

11. ANNEX III

11.1. Test Reporting Template for Pre-validation of SHE assay

TEST CHEMICAL		
Test Facility	Technician responsible	
Chemical Code	Experiment ID	
PREPARATION OF TEST CHEMICAL		
Solvent ______Culture Medium		______DMSO (0.2%)
Positive Control	Vehicle Control	solvent
CELL ISOLATE		
Cell batch	Supplier	
CELL CULTURE CONDITIONS		
Name of Medium	Supplier/ID	Lot No./Lab I.D.
Name of Serum	Supplier/ID	Lot No.
Serum Concentration	During Growth:	During Exposure:
TIMELINE		

12. ANNEX IV

12.1. Template for preparation of feeder cells

FEEDER CELLS

Project ID.: RING TEST CTA
Experiment No.:

Cell count
(mL cell susp. + mL trypan blue solution. 0.5%; Mallassez chamber, 1 big square)

count 1	count 2	mean	(x 4000) = cells / mL

Seeding in petridishes 60mm (cells / 2 mL = / mL)

Preparation of cell suspension for seeding	
x-irrad. cell suspension [mL]	in [mL] complete medium

12.2. Template for preparation of target cells

TARGET CELLS

Project ID.:
Experiment No.:

Count of cell suspension A :
(0.1 mL cell susp. + 0.1 mL trypan blue solution. 0.5%; Mallassez chamber, 1 big square)

count 1	count 2	mean	(x 8000) = cells / mL

Dilution of the cell suspension A : = cell suspension B

	Preparation of cell suspension for seeding				
	Nb of cells		Preparation		
	/dish	= /mL	Cell suspension B (mL)	Medium (mL)	Nb pools
Seeding 1					
Seeding 2					
Seeding 3					

13. ANNEX V

13.1. Template for Test Substance Preparation

Project ID.:
Experiment No.:

Test substance mixed before removal

Vehicle:	DMSO		
Dose [µg/mL cult.]	weighted sample / filled with vehicle		
	[mg]	[mL]	solubility

Test substance solution mixed with: shaker ☐; ultrasonic ☐; by pipetting ☐; for about min.

Dose [µg/mL cult.]	**Dilution**			
	[mL]	**from dose**	**+ vehicle [mL]**	**solubility**

Solubility: s = solution, e = emulsion; su = suspension

13.2. Template for Seeding and Treatment

TIME SCHEDULE, SEEDING AND TREATMENT

Project ID.:

Experiment No.:

(PT = pretest; MT = maintest)

		Seeding			Test substance		Fixing and dying
Dose [µg/mL cult.]	number of dishes	feeder cells 2 mL	target cells number	2 mL	Appli cation 4 mL	wash and refeed 8 mL	
		[time]					

13.3. Template for Treatment Conditions

TREATMENT CONDITIONS

Project ID.:

Experiment No.:
(PT = pretest; MT = maintest)

Dose [µg/mL culture]	pH-value	osmolarity [mOsm]	solubility in culture at treatment	
	after about 4 h		begin	end
Vehicle control				
Date / Sign				

s = soluble**; p** = precipitation

14. ANNEX VI

14.1. Template for Preliminary Cytotoxicity Assay

Project ID.:

Experiment No.:

Test groups	Colonies per dish	Plating efficiency %		Density / size of colonies	Feeder cells
Doses	Mean	Absolute	Relative		
Vehicle control					
µg/mL					
µg/mL					
µg/mL					
µg/mL					
µg/mL					
µg/mL					
µg/mL					
µg/mL					
µg/mL					
µg/mL					
µg/mL					
µg/mL					

P Precipitation
x Feeder cells present
a Feeder cells absent
\+ Cell density / size normal
++ Cell density / size slightly reduced
+++ Cell density / size greatly reduced

14.2. Template for Morphological Transformation – Raw data

Project-ID **Test item**
Experiment-No. **MT**

Test goup	Vehicle control																								B[a]P 5.0			Feeder control
				µg/mL																								
TCS																												
D/S																												
FC																												
	Col	MT	NSC	Col	MT	NSC	Col	MT	NSC	Col	MT	NSC	Col	MT	NSC	Col	MT	NSC	Col	MT	NSC	Col	MT	NSC	Col	MT	NSC	Col
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Date/sign:

Legend:

TCS	Target cells seeded	D / S	Colony density / size
Col	Colonies (all)	+	D/S normal
MT	Transformed colonies	+ +	D/S slightly reduced
NSC	Non-scorable colonies	+ ++	D/S greatly reduced
FC	Feeder cells	c	Contaminated
x	Feeder cells present	-	Not done / present
-	Feeder cells not present	B[a]P	Benzo[a]pyrene

14.3. Template for Morphological Transformation – Table Results

Project ID.:

Experiment No.:

Test groups	TCS	Colonies						Transformed Colonies		
		Total	Average	PE % abs.	PE % ± SD	RPE %	d / s	Total	MTF %	p value
Vehicle control										
µg/mL										
µg/mL										
µg/mL										
µg/mL										
µg/mL										
µg/mL										
µg/mL										
µg/mL										
B[a]P 5.0 µg/mL										

-	Not evaluated
P	Precipitation
B[a]P	Positive control benzo[a]pyrene
TCS	Target cells seeded
SD	Standard deviation
RPE	Relative plating efficiency
PE	Plating efficiency
MTF	Morphological transformation frequency
d / s	Density / size of colony
+	Cell density / size normal
++	Cell density / size slightly reduced
+++	Cell density / size greatly reduced
*	$p \leq 0.05$
**	$p \leq 0.01$

12.3 Repeated experiment: BASF 2,4-diaminotoluene

In the first experiment by BASF (TA1), 2,4 diaminotoluene was dissolved in DMSO. Table 36 shows the test concentrations which were evaluated based on solubility and cytotoxicity tests. The MTF values of the test chemical treated doses ranged from 0.15% to 1.08%. None of the test chemical concentrations had a significant increase in MTF compared to the VC (*p* • 0.05).
Only four scorable concentrations were available. In addition, based on the relatively weak positive response obtained with the PC and on the fact that results for one test chemical concentration (10 µg/ml) were in the same range as for the PC, the VMT considered this experiment worth repeating and asked BASF to repeat the experiment (see TA2, section 6.2.2.2).

Table 36: Transformation assay TA1 results from BASF, testing coded 2,4-diaminotoluene

BASF 2,4-diaminotoluene (µg/ml) TA1	Scorable colonies	RPE (%)	Colony density/size	Transformed colonies	MTF (%)	Fisher's exact test *p*-value (one-sided)
VC (DMSO 0.2%)	1121	100.0	+	5	0.45	-
5	1228	109.8	+	9	0.73	0.265
10	1295	112.9	+	14	1.08	0.061
20	1139	99.3	+	6	0.53	0.511
40	1120	65.1	++	9	0.80	0.211
60	670	41.0	++	1	0.15	0.940
80	-	-	+++	-	-	-
100	-	-	+++	-	-	-
PC	1287	112.2	+	15	1.17	0.041*

VC = Vehicle Control, PC = Positive Control, RPE = Relative Plating Efficiency, MTF = Morphological Transformation Frequency, + = normal density/size, ++ = slightly reduced density/size, +++= highly reduced density/size.
* = $p < 0.05$ (one-sided Fisher's exact test)