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PREVALIDATION STUDY REPORT

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The Inter-Organisation Programme for the Sound Management of Chemicals (IOMC) was established in 1995 following recommendations made by the 1992 UN Conference on Environment and Development to strengthen co-operation and increase international co-ordination in the field of chemical safety. The Participating Organisations are FAO, ILO, UNEP, UNIDO, UNITAR, WHO, World Bank and OECD. UNDP is an observer. The purpose of the IOMC is to promote co-ordination of the policies and activities pursued by the Participating Organisations, jointly or separately, to achieve the sound management of chemicals in relation to human health and the environment.

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FOREWORD

This document presents the pre-validation report of the cell transformation assay using syrian hamster embryonic (SHE) cells at pH 6.7. A pre-validation report is also available for the cell transformation assay using the same cells at pH 7.0 (see document N° 147 in the Series on Testing and Assessment).

This pre-validation report was submitted by the European Commission (ECVAM), and endorsed by the Working Group of National Coordinators of the Test Guidelines Programme at its meeting held in April 2011. It was declassified by the Joint Meeting of the Chemicals Committee and Working Party on Chemicals, Pesticides and Biotechnology (Joint Meeting) on 5 August 2011.

This document is published under the responsibility of the Joint Meeting.

30 JULY 2010

SYRIAN HAMSTER EMBRYONIC (SHE) CELL PH 6.7 CELL TRANSFORMATION ASSAY

PREVALIDATION STUDY REPORT

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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
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 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 contract research organisations (CROs) to screen for potential carcinogenicity as well as investigate mechanisms of carcinogenicity. CTAs are not used routinely for regulatory testing but they are often used for internal safety assessment of chemicals, drugs, *etc*. and are considered worthwhile for providing additional useful information to the prevailing tests that are used for assessing carcinogenic potential.

A recent detailed review paper (DRP) of the Organisation for Economic Co-operation and Development (OECD) on CTAs for the detection of chemical carcinogens (OECD, 2007) concluded that the performances 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 6.7 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 7.0 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 6.7 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 Organisation for Economic Development and Co-operation (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 useful approach for 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 contract research organisations (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 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 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 that these CTAs 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 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, 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 6.7 protocol.

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, the 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.* aromatic amines (*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 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.

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 cells) 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 primary SHE cells, (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 6.7 CTA (seven-day assay only) for the prediction of rodent carcinogenicity was reported for 88 chemicals as follows: concordance 74%, sensitivity 66%, specificity 85%, positive predictivity 88%, negative predictivity 62%, false positive 15%, false negative 33% (the proportion of carcinogens [prevalence] was 61%). 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 6.7 performed under the same conditions as those evaluated in the present study. However, two studies are worth mentioning. The first one has been published by LeBoeuf *et al*. and relates to the comparison of data produced in two laboratories. The authors concluded that the five chemicals (three chemical classes) tested in both laboratories showed reproducible results (LeBoeuf *et al*., 1989). In the second study, Engelhardt *et al*. (2004) tested 28 chemicals for (a) their ability to induce morphologically transformed colonies, (b) to determine how well the CTA performed at pH 6.7, and (c) if the assay could be transferred from an experienced laboratory to a naive one. The testing included 18 chemicals previously evaluated by the experienced laboratory and 10 new chemicals. The results obtained showed a concordance of 90% with the experienced laboratory results, demonstrating that with appropriate training the assay was successfully transferred.

The data collected in the OECD DRP for the assessment of the performance of the CTAs enabled an assessment of some measure of reproducibility beyond that suggested by the above studies (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 6.7 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 three 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 DRP, 2) the high costs and time to perform assays and 3) the limited funding and resources which could be made available by ECVAM. This allowed the evaluation of the CTA using the SHE pH 6.7 protocol, in three 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, USA)
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 an independent biostatistician (Sebastian Hoffmann - 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 three laboratories from Europe and the USA. The participating laboratories are listed below. Laboratories 1 and 3 had expertise with the assay and Laboratory 2 was new to the assay and thus served as a good test of assay transferability. Due to its expertise, Laboratory 1 acted as scientific lead laboratory, while Laboratory 2 had a role of administrative coordinator.

Laboratory 1 (Study Directors: Guenter Engelhardt* and Karl-Rainer Schwind)
(Scientific lead laboratory)
BASF Aktiengesellschaft
GV/TB
67056 Ludwigshafen,
Germany

*Dr Guenter Engelhardt retired in May 2006 and was replaced by Dr Markus Schulz.

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

2.3 Quality systems of the participating laboratories

The present study was conducted under Good Laboratory Practice (GLP)-like conditions by all laboratories and according to good scientific practice and good cell culture practice (OECD, 2004). All three participating laboratories 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), 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 was 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)	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 benzo(a)pyrene, 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 both a coded and non-coded chemical in this study phase. The laboratories were unaware of the fact that the same chemical served both purposes. 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 dimethyl sulfoxide (DMSO) should be used as the vehicle for all chemicals. Instructions were also sent regarding the order that the chemicals should be tested in. 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. Since the chemicals were coded, the laboratories did not know their identity and therefore all chemicals were treated as potential carcinogens.

The identity of the coded chemical used for the within-laboratory reproducibility and transferability was made known to the study directors at the VMT and study directors meeting of May 2006, after all the experiments of this phase were conducted.

For the between-laboratory reproducibility, the first statistical analysis was conducted before the decoding. The chemicals used were decoded during the VMT and study directors meeting of May 2007. Subsequent testing after this meeting requested by the VMT was performed non-coded.

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 three laboratories obtained for the same substance.

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

An important aspect of the initial phases of the study was the training of the laboratory personnel, including the harmonisation of scoring, the refinement of the protocols and the preparation of Standard Operating Procedures (SOPs). All laboratories participated to this training phase.

Following the preliminary phase of optimisation of the protocols, the transferability and 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 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 correct raw 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**	
08/2005	VMT kick-off meeting Berlin, Germany (in conjunction with the 5th World Congress on Alternative Methods)	The initial phase would assess : a) the transferability of the pH 6.7 protocol from the Scientific Lead Laboratory to Harlan CCR and BioReliance; b) the within-laboratory reproducibility, by testing benzo(a)pyrene as positive control and as a coded chemical.
09/2005	Training week Harlan CCR, Rossdorf, Germany	Technical staff and study directors from all participating laboratories agreed on the test method protocol and on the criteria for scoring the plates, using dishes treated with both the vehicle and positive controls.
05/2006	VMT and study directors meeting ECVAM, Ispra, Italy	Evaluation and discussion of results on standardisation of the protocol, within-laboratory reproducibility and transferability.
05/2007	VMT and study directors meeting ECVAM, Ispra, Italy	Evaluation and discussion of results on between-laboratory reproducibility and decoding of the chemicals. Additional testing requested by the VMT.
09/2007	VMT meeting ECVAM, Ispra, Italy	End of experimental part. Analysis of repeated experiments.
01/2009	VMT and study directors meeting ECVAM, Ispra, Italy	Final discussion.
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 underlying 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 6.7 CTA

To make the assay more accessible to more laboratories, the assay was modified to be performed at pH 6.7 by LeBoeuf *et al.* in the 1990s compared to the standard pH of 7.0-7.35 that had been used historically (LeBoeuf and Kerckaert, 1987). Advantages provided by pH 6.7 media include: (a) optimal growth for the SHE cells, (b) cells remain longer in a less differentiated state, and (c) cells take on a more fibroblastic spindle shape compared to growth in the standard pH media. These effects appear to be due to intracellular acidification (LeBoeuf *et al.*, 1992). The cells taking up a more fibroblastic appearance at pH 6.7, render the criss-cross pattern of growth in the morphologically transformed phenotype more apparent and easier to score. Optimised cell growth reduces variation in the use of different cell isolates, different lots of serum, and also increases the number of transformants in control and treated cultures which allows for the application of robust statistical methods. At the start of the ECVAM prevalidation study, most laboratories that were using the SHE CTA were trained in the pH 6.7 assay. Therefore, this protocol was selected for initial evaluation.

3.5 Protocol

The detailed test protocol used in this study 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 7.0, except that the pH of the medium used needs to be precisely adjusted to pH 6.7.

3.5.1 SHE cells

SHE cells were prepared by BioReliance (USA), following the protocol described in detail in Annex 12.2. The same isolate of cells was used by all laboratories during the complete prevalidation study.

For the isolate no. 062805, three female hamsters, 13-day pregnant, were received on 28 June 2005 from Harlan Sprague-Dawley and euthanised by CO_2 asphyxiation on the same day. The embryos were harvested, pooled, and SHE cells were prepared, grown *in vitro* to form sub-confluent monolayer cultures and subsequently harvested and cryopreserved in liquid nitrogen on 30 June 2005. Prior to using this isolate in the TA, 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.

3.5.2 Medium

Initially, Harlan CCR and BioReliance used the same DMEM-L (Dulbecco's Modified Eagle Medium with LeBoeuf's modification) culture medium (not the same batch) from Quality Biologicals, USA. BASF used DMEM-L medium from Biochrom, Berlin (catalogue no. FZ 9995). For the between-laboratory reproducibility assessment, all laboratories used the same batch of medium from Quality Biologicals, USA.

3.5.3 Serum

All the laboratories used the same batch of foetal bovine serum (FBS) (Hyclone, catalogue no. SH 30071-03, lot no. APB 20666) for the whole study.

A SHE cell isolate that had been shown to work appropriately in a previous study was used by Bioreliance to test this batch of FBS prior use:
- The target cell number producing 25 to 45 colonies per 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, 5.0 µg/ml of benzo(a)pyrene) 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.

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 are 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 are treated with 4 ml of complete medium containing the test chemical. The cells are exposed to the test chemical for seven days. At the end of the exposure period the medium is removed and the cells are washed with phosphate-buffered saline (PBS), fixed with absolute ethanol and stained with 10% aqueous Giemsa. After rinsing with tap water, the dishes are air dried before being scored. Each dish is coded and scored blindly. The colonies are examined under a stereomicroscope for scoring normal or morphologically transformed phenotypes. The morphologically transformed cells are characterised by a spindle shape, an increased nuclear/cytoplasm ratio and a higher basophilic affinity. These cells have a criss-cross orientation pattern and may be multilayered compared to normal cells.

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

At least five concentrations per chemical-treated group are scored, so typically seven to eight concentrations are 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 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 is non-toxic, then at least five concentrations are 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 is within two-times the visible solubility limit in complete medium. For toxic and insoluble test chemicals, the highest dose level tested causes 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 the 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 led to a statistically significant increase in morphological transformation compared to the VC (significance level: $p < 0.05$).

3.5.7 Assay acceptance criteria

The following assessment criteria were discussed and agreed upon 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. However the average number of colonies per plate should not be less than 25),
- There should be 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 in the case of positive results with > 45 colonies per dish),
- Transformation frequency in the negative (untreated and vehicle) controls 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 the experience of the participating laboratories and on the literature (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 non-coded and then 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 assay was further evaluated.

Figure 1-Figure 6 and Table 3-Table 5 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 reduced 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.

The VC (DMSO 0.2%) gave transformation frequencies within the expected range (≤0.6%) for the SHE cells under the assay conditions employed: 0.37% (BASF), 0.27% (Harlan CCR) and 0.47% (BioReliance) (Figure 1).

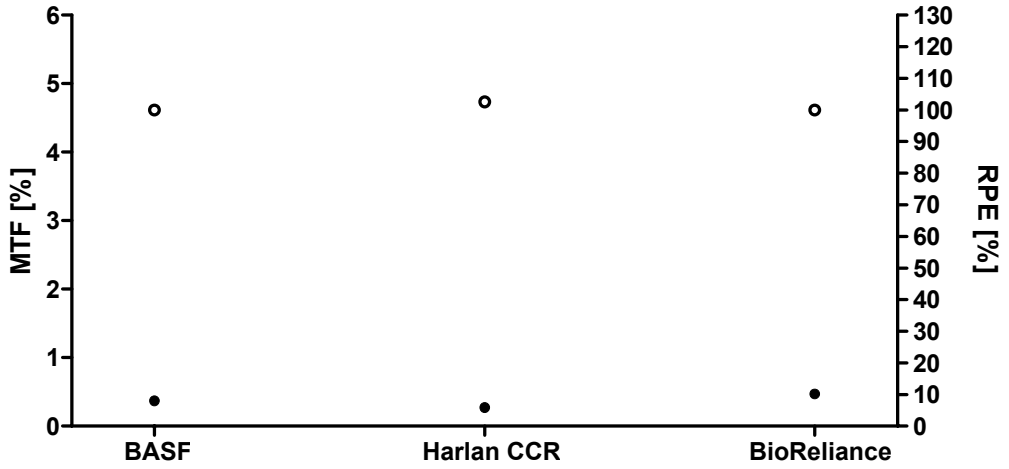

Figure 1: Morphological Transformation Frequency (MTF [•]) and Relative Plating Efficiency (RPE [◦]) of the vehicle control (0.2% DMSO) in all laboratories testing non-coded benzo(a)pyrene

The PC chemical (non-coded benzo(a)pyrene) led to the expected increase in MTF: 2.47% (BASF), 2.09% (Harlan CCR) and 2.29% (BioReliance), which was in a similar range in all laboratories (Figure 2).

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.

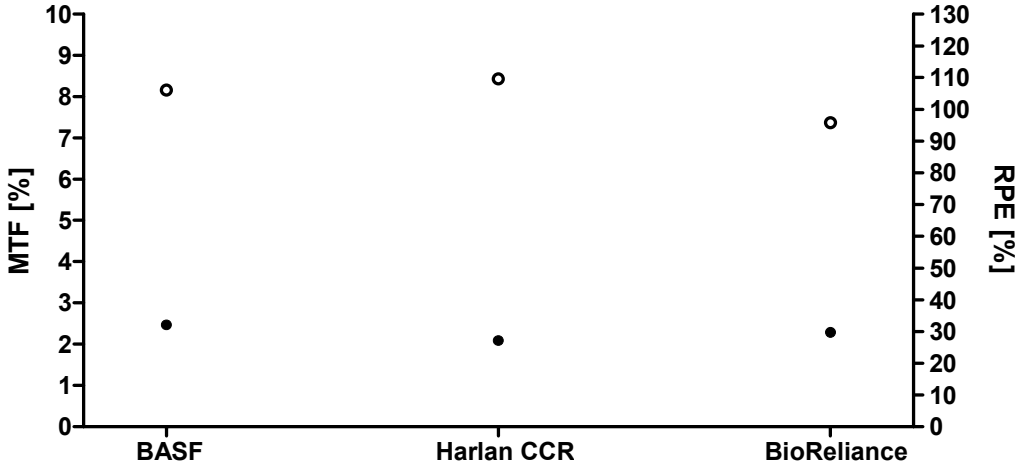

Figure 2: Morphological Transformation Frequency (MTF [•]) and Relative Plating Efficiency (RPE [◦]) of the positive control (5 µg/ml benzo(a)pyrene) in all laboratories testing non-coded benzo(a)pyrene

4.1 Dose-range finding test - coded benzo(a)pyrene

Each laboratory carried out DRF tests to determine the experimental doses. Figure 3 shows the results of the DRF tests with coded benzo(a)pyrene. Benzo(a)pyrene was dissolved in DMSO. It can be observed that at the concentrations tested, limited or no cytotoxicity was observed, as expected based on literature. Therefore solubility was the criterion to determine the top dose.

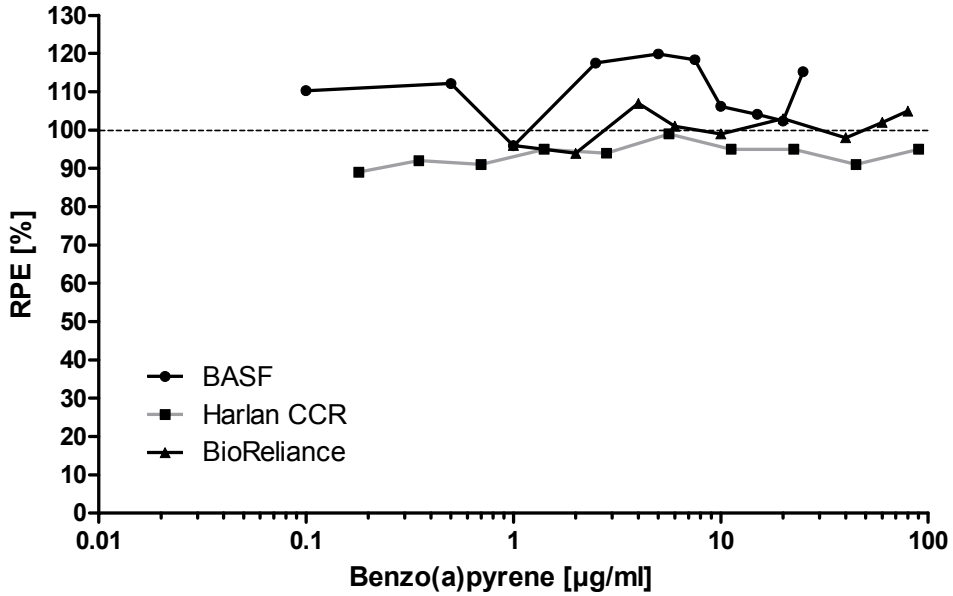

Figure 3: Relative Plating Efficiency (RPE) compared to vehicle control in all laboratories testing coded benzo(a)pyrene

4.2 Transformation Assay - coded benzo(a)pyrene

4.2.1 BASF

Based upon the results of the preliminary DRF test, the doses selected for evaluation of induction of morphological transformation ranged from 0.3 to 20 µg/ml. The MTF values ranged from 1.73% to 2.33%. The highest test chemical concentration of 20 µg/ml showed signs of precipitate in the treatment media. All test chemical concentrations induced significant increases in MTF compared to the VC ($p < 0.01$). The results are shown in Table 3.

Table 3: 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%)	1639	100	+	6	0.37	-
0.3	1501	95.5	+	35	2.33	< 0.0001**
0.6	1663	101.6	+	35	2.10	< 0.0001**
1.2	1698	104.2	+	33	1.94	< 0.0001**
2.5	1710	106.1	+	38	2.22	< 0.0001**
5.0	1672	102.1	+	29	1.73	< 0.0001**
10	1652	104.7	+	30	1.82	< 0.0001**
20	1769	107.8	+	34	1.92	< 0.0001**
5.0 PC	1738	106.0	+	43	2.47	< 0.0001**

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)

4.2.2 Harlan CCR

No toxicity was observed in the preliminary DRF test, therefore a concentration of 45 µg/ml of benzo(a)pyrene, which was observed to precipitate in the aqueous cell culture medium, was chosen as the top concentration for the TA. The doses selected for evaluation of induction of morphological transformation ranged from 2.81 to 45 µg/ml and the MTF values ranged from 0.89% to 1.97%. All test chemical concentrations induced significant increases in MTF compared to the VC ($p < 0.01$). The results are shown in Table 4.

Table 4: 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%)	2238	100	+	6	0.27	-
2.81	2347	105.2	+	38	1.62	<0.0001**
5.63	2138	95.3	+	19	0.89	0.0052**
11.25	2227	99.0	+	40	1.80	<0.0001**
22.5	2445	109.3	+	44	1.80	<0.0001**
45	2644	117.5	+	52	1.97	<0.0001**
5.0 PC	2391	106.8	+	50	2.09	<0.0001**

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)

4.2.3 BioReliance

The test doses ranged from 2.0 to 40 µg/ml (Table 5). Precipitation of benzo(a)pyrene in the aqueous cell culture medium was noted at the two highest concentrations employed. As PCs, one dose of benzo(a)pyrene obtained from ECVAM along with the standard benzo(a)pyrene from BioReliance were tested at the concentration of 5.0 µg/ml each. The MTF values for the test chemical concentrations ranged from 2.17% to 5.03%. All test chemical concentrations induced significant increases in MTF compared to the VC ($p < 0.01$).

Table 5: 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%)	1689	100	+	8	0.47	-
2.0	1426	84.6	+	31	2.17	<0.001**
4.0	1353	80.6	+	46	3.40	<0.001**
6.0	1311	78.5	++	58	4.42	<0.001**
8.0	1160	69.1	++	41	3.53	<0.01**
10	1205	72.0	++	56	4.65	<0.001**
20 #	1193	71.3	++	60	5.03	<0.001**
40 #	1473	87.4	+	38	2.58	<0.001**
PC (B)	1592	94.1	+	24	1.51	0.004**
PC (E)	1616	95.8	+	37	2.29	<0.001**

# = Precipitation seen in culture medium
VC = Vehicle Control, PC = Positive Control, B = BioReliance benzo(a)pyrene, E = ECVAM benzo(a)pyrene, RPE = Relative Plating Efficiency, MTF = Morphological Transformation Frequency, + = normal density/size, ++ = slightly reduced density/size.
** = $p < 0.01$ (one-sided Fisher's exact test)

4.2.4 Concurrent cytotoxicity (Relative Plating Efficiency)

Cytotoxicity of benzo(a)pyrene was evaluated by RPE assessment in all laboratories (Figure 4). No cytotoxicity was observed by BASF and Harlan CCR, while moderate cytotoxicity was observed by BioReliance.

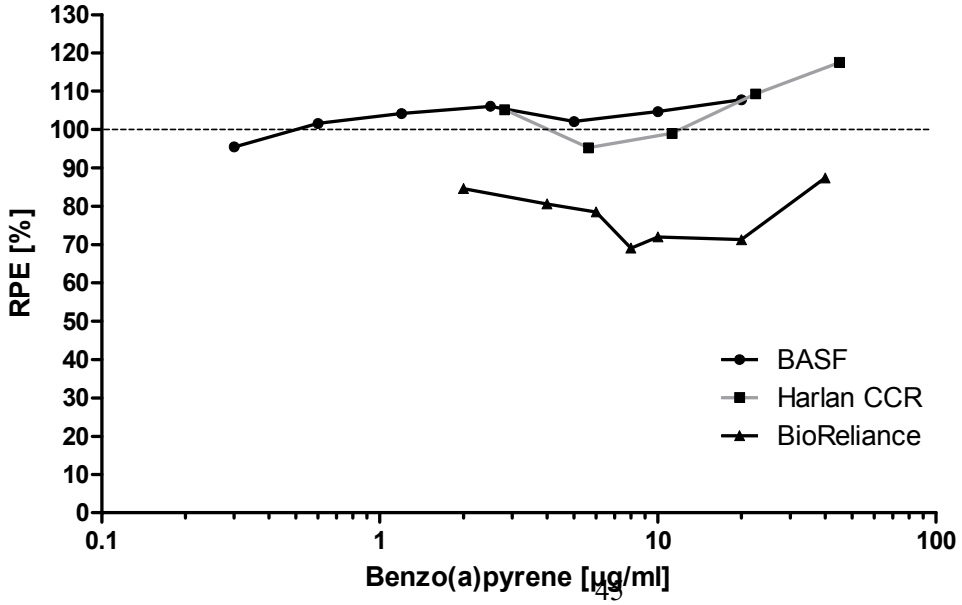

Figure 4: Relative Plating Efficiency (RPE) compared to vehicle control in all laboratories testing coded benzo(a)pyrene

4.2.5 Morphological transformation frequency

MTF results with benzo(a)pyrene are shown in Figure 5. A statistically significant increase in MTF compared to the VC was observed in all laboratories at all concentrations tested. BioReliance found a higher number of transformed foci at higher concentrations compared to the other laboratories.

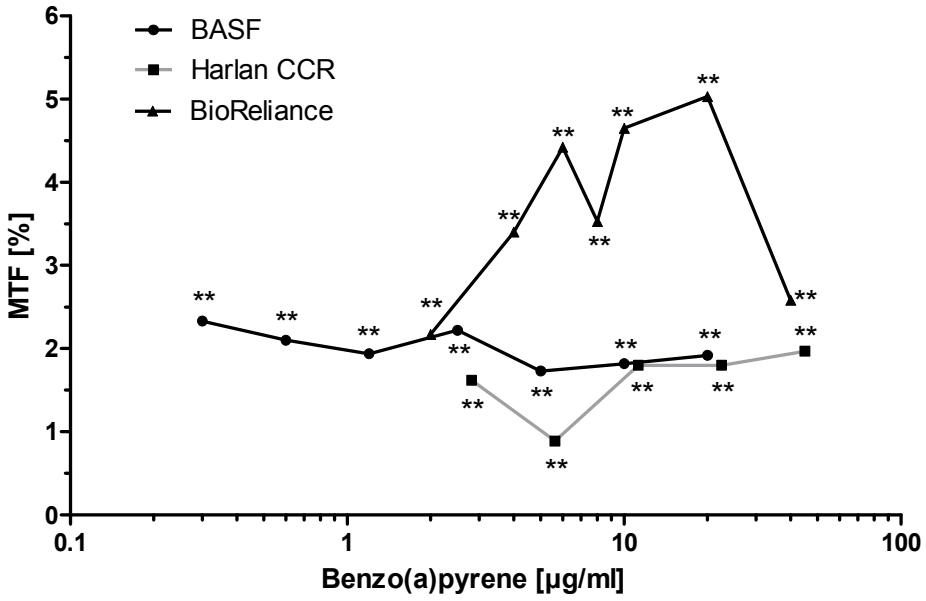

Figure 5: Morphological Transformation Frequency (MTF) shown as a percentage of transformed colonies compared to scorable colonies, for all laboratories testing coded benzo(a)pyrene.
** = $p < 0.01$ (one-sided Fisher's exact test)

4.2.6 Reproducibility: non-coded versus coded benzo(a)pyrene

TA results for coded and non-coded benzo(a)pyrene are summarised in Figure 6. Comparison of MTF and RPE data within and between the laboratories shows a good reproducibility of the assay. Only the non-coded benzo(a)pyrene from BioReliance gave a higher MTF value, which was correlated to increased cytotoxicity.

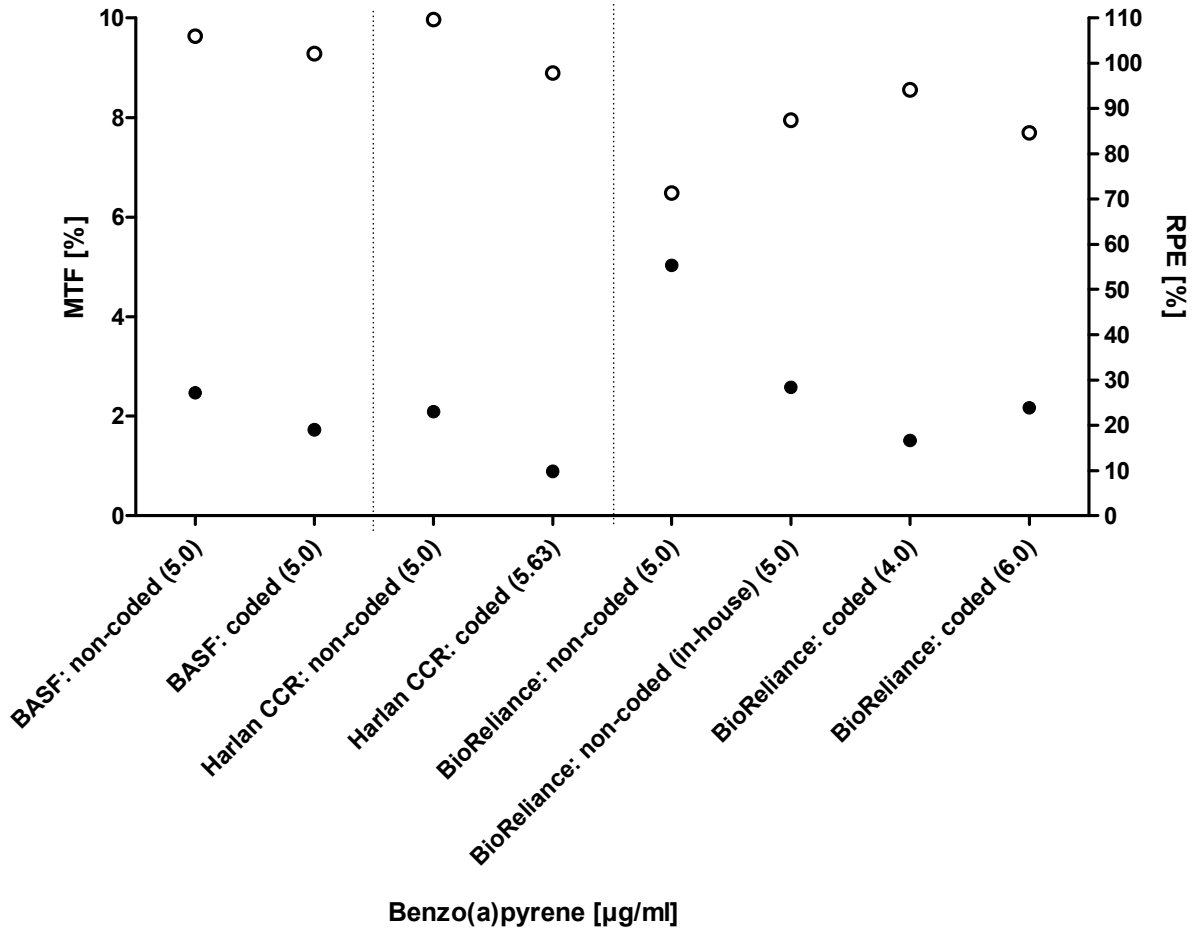

Figure 6: Morphological Transformation Frequency (MTF [•]) and Relative Plating Efficiency (RPE [◦]) of the positive control (benzo(a)pyrene) and coded test chemical (benzo(a)pyrene). The numbers in brackets indicate the treatment concentration in µg/ml.

4.2.7 Acceptance criteria and assessment

All acceptance criteria were met in all three laboratories (Table 6). The number of colonies in the TA by Harlan CCR was higher than 45. However since the chemical was positive, this was considered acceptable.

Table 6: Acceptance criteria and assessment of benzo(a)pyrene results

Benzo(a)pyrene			
Criteria	**Laboratory**		
	BASF	**Harlan CCR**	**BioReliance**
Number of scorable colonies per treatment group ≥1000, or <1000 if positive result	**yes** (≥1501)	**yes** (≥2138)	**yes** (≥1160)
Average number of 25-45 colonies per dish or >45 colonies if positive result or <25 colonies if negative result	**yes** (40.0-45.0)	**yes** (54.3-66.9)	**yes** (29.3-42.3)
Plating Efficiency of vehicle control >20%	**yes** (37.7%)	**yes** (57.0%)	**yes** (35.3%)
Morphological Transformation Frequency of vehicle control <0.6%	**yes** (0.37%)	**yes** (0.27%)	**yes** (0.47%)
Number of scorable concentrations ≥5	**yes** (7)	**yes** (5)	**yes** (7)
Fisher's test *p*-value of positive control <0.05	**yes** (<0.0001)	**yes** (<0.0001)	**yes** (0.004[#], <0.001[##])
Fulfilment of all assay acceptance criteria	**YES**	**YES**	**YES**
Assessment	+	+	+

[#] for BioReliance positive control
[##] for ECVAM positive control

4.3 Conclusion of the Validation Management Team on Module 2

The data generated showed a good within-laboratory reproducibility for the coded and non-coded benzo(a)pyrene. Moreover the results provided an initial indication of the between-laboratory reproducibility for benzo(a)pyrene and demonstrated that the method had been successfully transferred to all laboratories. The lack of a dose-response increase in MTF and cytotoxicity upon treatment with benzo(a)pyrene was expected considering that solubility limits had been reached. Results from this phase of the study agreed with published data for benzo(a)pyrene (LeBoeuf *et al*., 1987; Engelhardt *et al*., 2004).

The VMT agreed that the data generated were reproducible. Therefore the participating laboratories could proceed to the between-laboratory reproducibility assessment phase of the prevalidation study.

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.

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 week was held at Harlan CCR (Rossdorf, Germany) on 27-30 September 2005. Representatives from all laboratories involved (including technical staff and study directors) 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 week was extremely useful for harmonising the procedures among the laboratories for the prevalidation experiments.

The morphology of the transformed colonies was also discussed and agreed upon prior to starting the experiments. As part of this prevalidation exercise, a photo catalogue was produced by the participating laboratories 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.

5.3 Conclusion of the Validation Management Team on Module 3

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) were maintained as were used for the within-laboratory reproducibility. 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 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 7, Figure 10, Figure 13, Figure 16 and Figure 19). Subsequently, the complete TA including concurrent cytotoxicity tests (Figure 8, Figure 11, Figure 14, Figure 17 and Figure 20) and MTF assay were performed (Figure 9, Figure 12, Figure 15, Figure 18 and Figure 21).

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 7 shows the results of the DRF tests with anthracene. At the concentrations tested, no cytotoxicity was observed. However, cell proliferation was seen in all laboratories.

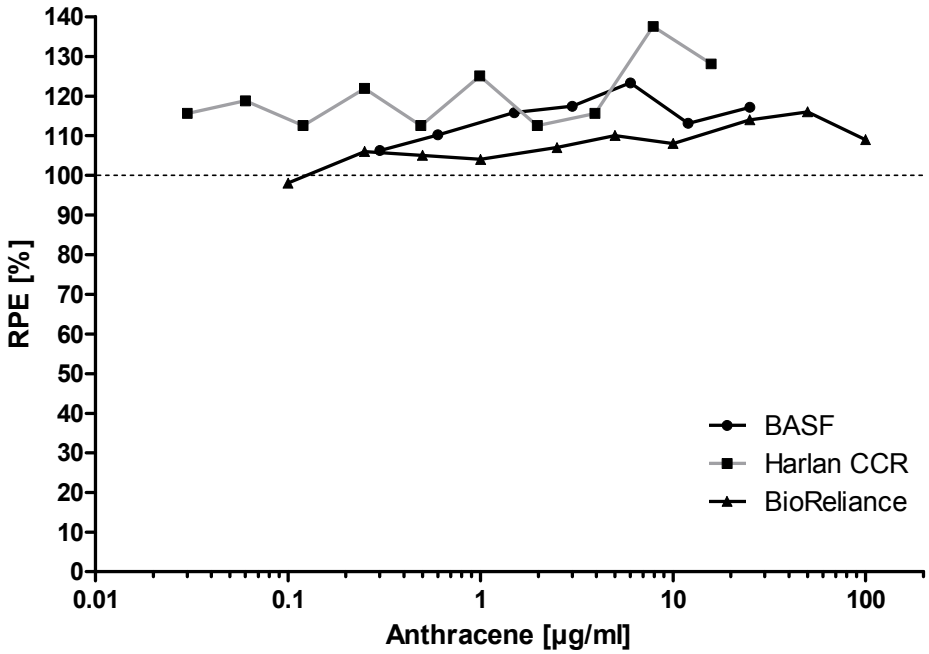

Figure 7: Relative plating efficiency (RPE) compared to vehicle control in all laboratories testing coded anthracene, for the Dose-Range Finding

6.1.2 Transformation Assay

VCs gave transformation frequencies within the expected range (≤0.6%): 0.24% (BASF), 0.06% (Harlan CCR) and 0.42% (BioReliance). The PC chemical benzo(a)pyrene led to the expected increase in morphologically transformed colonies: 0.99% (BASF), 1.58% (Harlan CCR) and 1.28% (BioReliance). Coded anthracene was tested to precipitating concentrations, in all laboratories, as described in the protocol (Annex 12.2).

6.1.2.1 BASF

Anthracene was dissolved in DMSO. The concentrations and the top dose of 25 µg/ml were selected on the basis of the DRF test and the solubility of the chemical. The MTF values of the test chemical doses ranged from 0.22 to 0.39% and the VC value was 0.24%. None of the test chemical concentrations induced a significant increase in MTF compared to the VC ($p \geq 0.05$). The results evaluated by BASF are shown in Table 7.

Table 7: 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%)	1243	100.0	+	3	0.24	-
0.3	1280	105.1	+	5	0.39	0.3792
0.6	1321	107.9	+	3	0.23	0.6845
1.5	1364	112.5	+	5	0.37	0.415
3	1225	103.0	+	2	0.16	0.8082
6	1388	113.0	+	2	0.14	0.8453
12	1339	112.0	+	3	0.22	0.6906
25[#]	1424	115.3	+	5	0.35	0.4396
PC	1210	102.7	+	12	0.99	0.0152*

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

6.1.2.2 Harlan CCR

Anthracene was dissolved in DMSO. The concentrations and the top dose of 16 µg/ml were selected on the basis of the DRF test and the solubility of the chemical. The MTF values of the test chemical doses ranged from 0.14 to 0.37% and the VC value was 0.06%. None of the test chemical concentrations induced a significant increase in MTF compared to the VC ($p \geq 0.05$). The results evaluated by Harlan CCR are shown in Table 8.

Table 8: 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%)	1669	100	+	1	0.06	-
0.5	1522	91.4	+	3	0.20	0.2786
1	1477	88.8	+	2	0.14	0.4543
2	1634	98.1	+	3	0.18	0.3045
4	1603	95.2	+	6	0.37	0.0560
8[#]	1606	96.9	+	5	0.31	0.1004
16[##]	1534	92.4	+	4	0.26	0.1621
PC	1519	91.7	+	24	1.58	0.0001**

[#] = Precipitation seen microscopically in culture medium, [##] = Precipitation seen in culture 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)

6.1.2.3 BioReliance

Anthracene was dissolved in DMSO. The concentrations and the top dose of 100 µg/ml were selected on the basis of the DRF test and the solubility of the substance. The MTF values of the test chemical doses ranged from 0.36 to 0.83% and the VC value was 0.42%. None of the test chemical concentrations induced a significant increase in MTF compared to the vehicle control ($p \geq 0.05$). The results evaluated by BioReliance are shown in Table 9.

Table 9: 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%)	1686	100	+	7	0.42	-
2.5	1643	97.5	+	6	0.37	0.6934
5	1655	98.4	+	6	0.36	0.6981
10	1708	101.4	+	9	0.53	0.4118
25	1780	105.7	+	11	0.62	0.2775
50[#]	1568	93.5	+	13	0.83	0.0991
100[#]	1641	97.8	+	6	0.37	0.6926
PC	1645	98	+	21	1.28	0.0051**

[#] = Precipitation seen in culture media
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 Concurrent cytotoxicity (Relative Plating Efficiency)

Cytotoxicity of anthracene was evaluated by RPE assessment (Figure 8). Anthracene was not found cytotoxic in any laboratory.

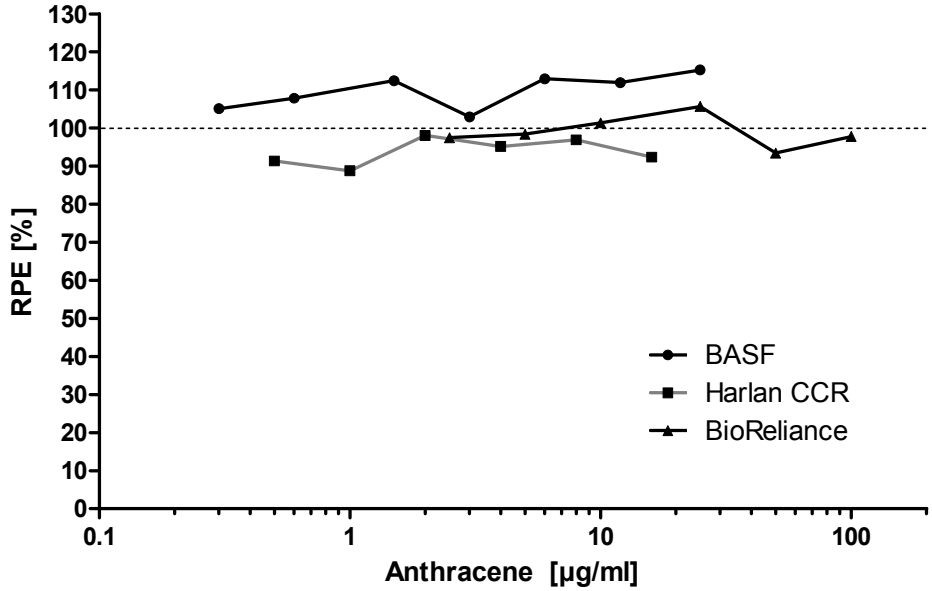

Figure 8: Relative plating efficiency (RPE) compared to vehicle control in all laboratories testing coded anthracene

6.1.2.5 Morphological transformation frequency

MTF results with anthracene are shown in Figure 9. Anthracene did not induce a statistically significant increase in morphological transformation in any of the laboratories.

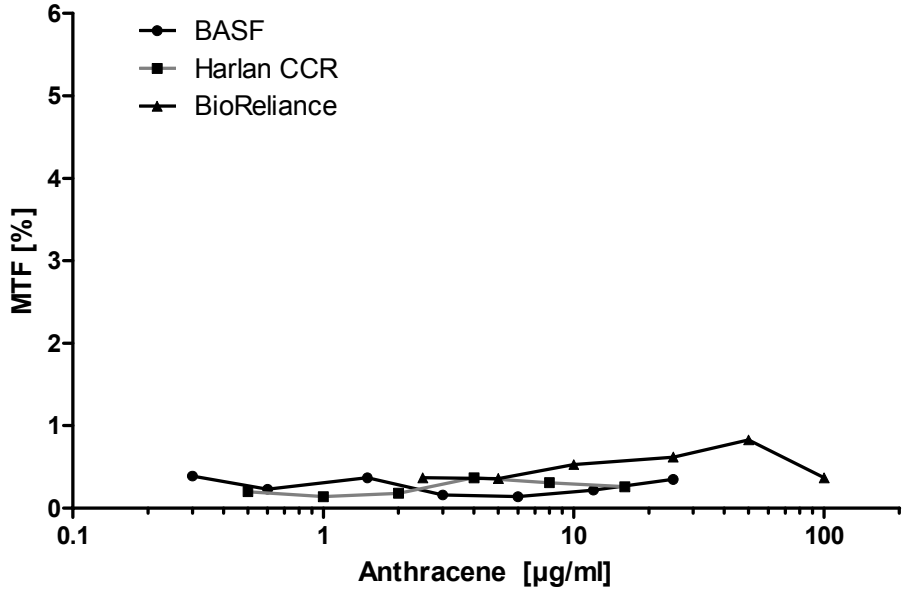

Figure 9: Morphological Transformation Frequency (MTF) shown as a percentage of transformed colonies compared to total colonies in all laboratories testing coded anthracene

6.1.2.6 Acceptance criteria and assessment

All acceptance criteria were met in all laboratories. Anthracene was assessed to be negative by all laboratories (Table 10).

Table 10: Acceptance criteria and assessment of anthracene results

Anthracene			
Criteria	**Laboratory**		
	BASF	**Harlan CCR**	**BioReliance**
Number of scorable colonies per treatment group ≥1000 or <1000 if positive result	**yes** (≥1210)	**yes** (≥1477)	**yes** (≥1568)
Average number of 25-45 colonies per dish or >45 colonies if positive result or <25 colonies if negative result	**yes** (31.5-36.3)	**yes** (37.1-41.0)	**yes** (39.5-44.6)
Plating Efficiency of vehicle control >20%	**yes** (28.6%)	**yes** (49.1%)	**yes** (35.1%)
Morphological Transformation Frequency of vehicle control <0.6%	**yes** (0.24%)	**yes** (0.06%)	**yes** (0.42%)
Number of scorable concentrations ≥5	**yes** (7)	**yes** (6)	**yes** (6)
Fisher's test *p*-value of positive control <0.05	**yes** (0.0152)	**yes** (0.0001)	**yes** (0.00506)
Fulfilment of all assay acceptance criteria	**YES**	**YES**	**YES**
Assessment	**-**	**-**	**-**

6.1.3 Conclusion

Treatment with anthracene did not produce a statistically significant increase in morphologically transformed colonies in any laboratory and at any dose tested. As such, anthracene is considered to be non-transforming in the SHE pH 6.7 CTA. These results agree with published data (LeBoeuf *et al*., 1996).

6.2 2,4-Diaminotoluene

6.2.1 Dose-range finding test

Figure 10 shows the results of the DRF tests with 2,4-diaminotoluene. All laboratories observed a dose-dependent cytoxicity induced by 2,4-diaminotoluene at the concentrations tested.

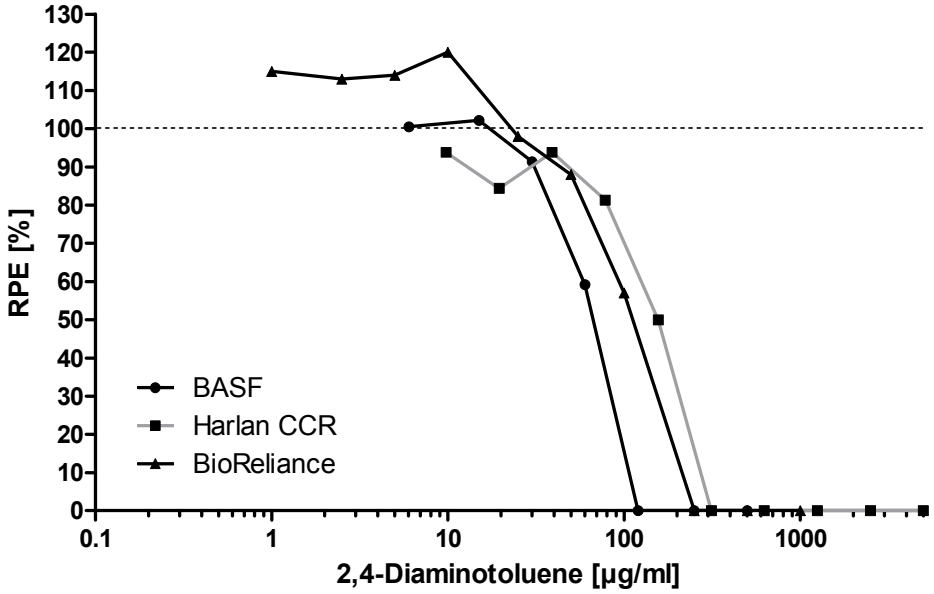

Figure 10: Relative Plating Efficiency (RPE) compared to vehicle control in all laboratories testing coded 2,4-diaminotoluene, for the Dose-Range Finding

6.2.2 Transformation assay

VCs gave transformation frequencies within the expected range (≤0.6%): 0.39% (BASF), 0.14% (Harlan CCR) and 0.45 % (BioReliance). The PC chemical benzo(a)pyrene led to the expected increase in morphologically transformed colonies: 1.38% (BASF), 2.06% (Harlan CCR) and 2.05% (BioReliance).

6.2.2.1 BASF

2,4-Diaminotoluene was dissolved in DMSO. The concentrations were selected on the basis of the DRF test and evaluated by BASF. The MTF values of the test chemical doses ranged from 0.29 to 2.19% 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 11). At the highest concentration no significant increase in MTF was observed, most probably due to the cytotoxicity caused by the test chemical.

Table 11: Transformation assay results from BASF, testing coded 2,4-diaminotoluene

BASF 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%)	1022	100.0	+	4	0.39	-
10	1522	125.2	+	8	0.53	0.433
20	1454	118.6	+	8	0.55	0.4021
30	1380	115.0	+	11	0.80	0.1623
40	1060	92.7	+	14	1.32	0.0183*
50	1048	97.9	+	23	2.19	0.0002**
60	1355	80.4	+	22	1.62	0.0027**
70	1018	52.3	+++	3	0.29	0.7722
PC	1303	108.7	+	18	1.38	0.0105*

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)

6.2.2.2 Harlan CCR

Harlan CCR initially tested 2,4-diaminotoluene using DMEM-L as the vehicle and the results of this experiment (TA1) are shown in Annex 12.3.1. Since all laboratories were requested to use DMSO as vehicle, this laboratory was asked to repeat the experiment using the correct vehicle. Table 12 shows the results of the TA using DMSO as vehicle (TA2). The MTF values of the test chemical doses ranged from 1.06 to 2.55% and the VC value was 0.14%. All the test chemical concentrations induced a significant increase in MTF compared to the VC ($p < 0.05$), with a dose-dependent effect.

Table 12: Transformation assay TA2 results from Harlan CCR, testing coded 2,4-diaminotoluene

Harlan CCR 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%)	1400	100	+	2	0.14	-
10	1513	108.3	+	16	1.06	0.0011**
20	1323	94.5	+	15	1.13	0.0008**
40	1138	81.6	++	19	1.67	<0.0001**
50	1097	78.5	++	20	1.82	<0.0001**
60	1097	59.5	++	27	2.46	<0.0001**
70	1072	57.6	+++	25	2.33	<0.0001**
80	1058	37.9	+++	27	2.55	<0.0001**
PC	1407	100.5	+	29	2.06	<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.
** = $p < 0.01$ (one-sided Fisher's exact test)

6.2.2.3 BioReliance

2,4-Diaminotoluene was dissolved in DMSO. The concentrations were selected on the basis of the DRF test, and evaluated by BioReliance (Table 13). The MTF values of the test chemical doses ranged from 1.68 to 3.06% and the VC value was 0.45%. All the test chemical concentrations induced a significant increase in MTF compared to the VC ($p < 0.05$).

Table 13: 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%)	1324	100	+	6	0.45	-
5	1489	112.8	+	25	1.68	0.0012**
10	1372	103.9	+	42	3.06	<0.0001**
25	1392	105.6	+	40	2.87	<0.0001**
50	1297	98.5	+	27	2.08	0.0001**
100	1104	56.2	++	19	1.72	0.0018**
PC	1512	114.3	+	31	2.05	0.0001**

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

6.2.2.4 Concurrent cytotoxicity (Relative Plating Efficiency)

2,4-Diaminotoluene cytotoxicity was evaluated measuring RPE (Figure 11) and this chemical was shown to be dose-dependently cytotoxic in all laboratories.

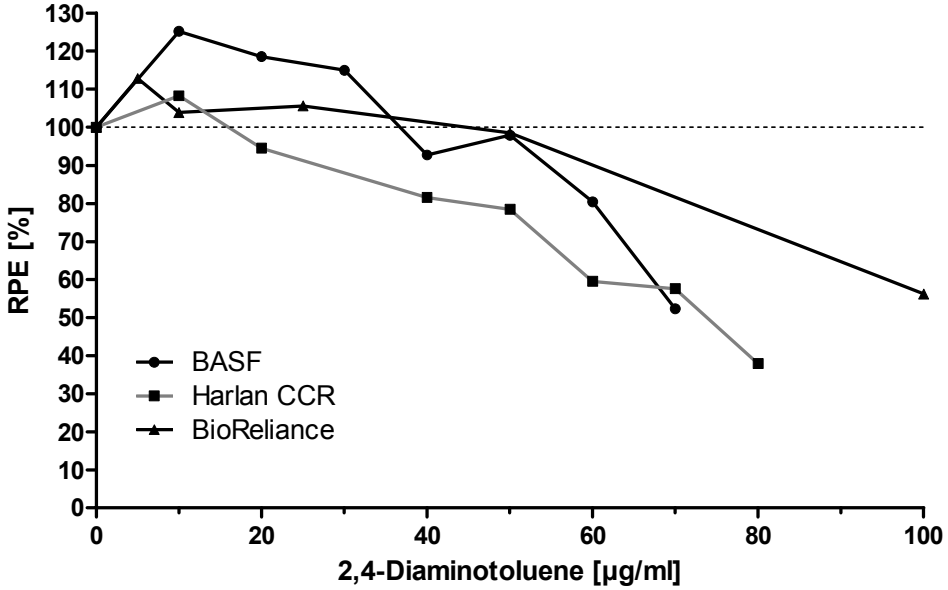

Figure 11: Relative plating efficiency (RPE) compared to vehicle control in all laboratories testing coded 2,4-diaminotoluene

6.2.2.5 Morphological transformation frequency

MTF results with 2,4-diaminotoluene are shown in Figure 12. 2,4-Diaminotoluene induced a statistically significant increase in morphological transformation compared to the VC in all laboratories, with a clear dose-dependent response for Harlan CCR. In Harlan CCR and BioReliance a significant increase was already seen at low concentrations, while in BASF it was observed from 40 µg/ml only. Moreover, some cytotoxic effects were observed by BASF at the two highest concentrations.

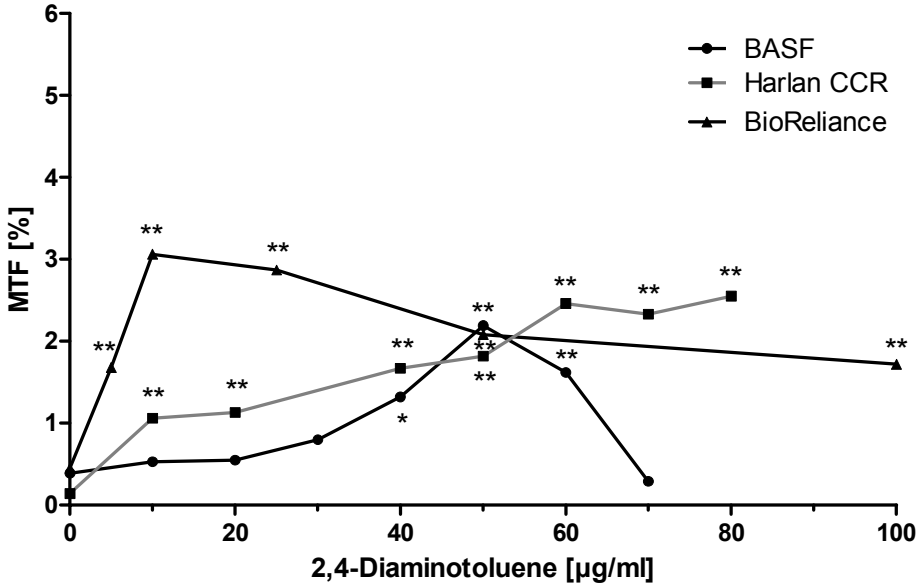

Figure 12: Morphological Transformation Frequency (MTF) shown as a percentage of transformed colonies compared to total colonies for all laboratories testing 2,4-diaminotoluene

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

6.2.2.6 Acceptance criteria and assessment

All acceptance criteria were met in all laboratories. 2,4-Diaminotoluene was assessed to be positive by all laboratories (Table 14).

Table 14: Acceptance criteria and assessment of 2,4-diaminotoluene results

2,4-Diaminotoluene			
Criteria	**Laboratory**		
	BASF	**Harlan CCR**	**BioReliance**
Number of scorable colonies per treatment group ≥1000 Or <1000 if positive result	**yes** (≥1018)	**yes** (≥1058)	**yes** (≥1104)
Average number of 25-45 colonies per dish or >45 colonies if positive result or <25 colonies if negative result	**yes** (28.8-39.0)	**yes** (26.6-38.0)	**yes** (27.9-37.8)
Plating Efficiency of vehicle control >20%	**yes** (28.3%)	**yes** (39.0%)	**yes** (27.6%)
Morphological Transformation Frequency of vehicle control <0.6%	**yes** (0.39%)	**yes** (0.14%)	**yes** (0.45%)
Number of scorable concentrations ≥5	**yes** (7)	**yes** (7)	**yes** (5)
Fisher's test *p*-value of positive control <0.05	**yes** (0.0105)	**yes** (<0.0001)	**yes** (0.0001)
Fulfilment of all assay acceptance criteria	**YES**	**YES**	**YES**
Assessment	+	+	+

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 6.7 CTA. These results agree with published data (LeBoeuf *et al*., 1996; Engelhardt *et al*., 2004).

6.3 3-Methylcholanthrene

6.3.1 Dose-range finding test

Figure 13 shows the results of the DRF tests with 3-methylcholanthrene. 3-Methylcholanthrene showed limited cytotoxicity at the concentrations tested. BASF did not perform DRF because a dose range was suggested by the VMT, which was thus considered sufficient by this laboratory for selecting the adequate doses.

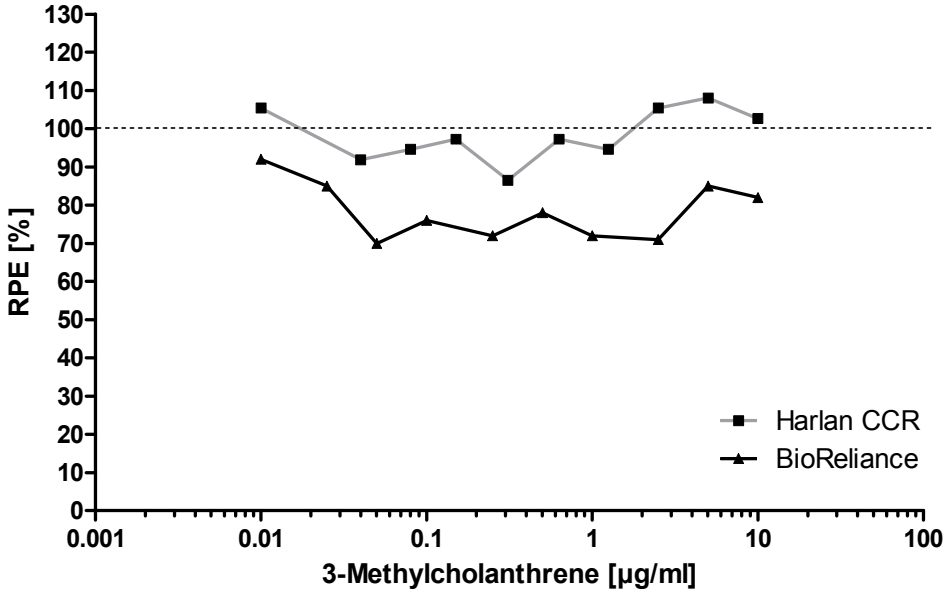

Figure 13: Relative plating efficiency (RPE) compared to vehicle control in all laboratories testing coded 3-methylcholanthrene, for the Dose-Range Finding

6.3.2 Transformation assay

VCs gave transformation frequencies within the expected range (≤0.6%): 0.17% (BASF), 0.28% (Harlan CCR) and 0.44% (BioReliance). The PC chemical benzo(a)pyrene led to the expected increase in morphologically transformed colonies: 2.52% (BASF), 2.30% (Harlan CCR) and 1.99% (BioReliance). The highest dose tested was 10 µg/ml, as suggested by the VMT.

6.3.2.1 BASF

BASF initially tested 3-methylcholanthrene using DMEM-L as the vehicle and thus had used a concentration range much higher than that suggested by the VMT. The results of this experiment (TA1) are shown in Annex 12.3.2. Since all laboratories were requested to use DMSO as vehicle, BASF was asked to repeat the experiment using the recommended vehicle (TA2). Table 15 summarises the data from the experiment reported in "Amendment no. 1 to the report" by BASF (26 September 2007). This amendment describes the experimental conditions and results of the test with 3-methylcholanthrene in DMSO (the recommended vehicle), using the appropriate concentration range. The MTF values of the test chemical doses ranged from 0.96 to 1.94%. All the test chemical concentrations induced significant increases in MTF compared to the VC ($p < 0.05$).

Table 15: Transformation assay TA2 results from BASF, testing coded 3-methylcholanthrene

BASF 3-methylcholanthrene (µg/ml) TA2	Scorable colonies	RPE (%)	Colony density/size	Transformed colonies	MTF (%)	Fisher's exact test *p*-value (one-sided)
VC (DMSO 0.2%)	1763	100	+	3	0.17	-
0.25	1669	94.9	+	16	0.96	0.0015**
0.5	1743	96.8	+	19	1.09	0.0004**
1	1650	91.2	+	32	1.94	<0.0001**
2.5	1632	93.7	+	27	1.65	<0.0001**
5	1593	91.0	+	30	1.88	<0.0001**
10	1700	95.3	+	28	1.65	<0.0001**
PC	1625	92.8	+	41	2.52	<0.0001**

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)

6.3.2.2 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 16. The MTF values of the test chemical doses ranged from 0.85 to 2.21%. All the test chemical concentrations induced significant, dose-dependent increases in MTF compared to the VC ($p < 0.05$).

Table 16: 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%)	1422	100	+	4	0.28	-
0.3	1405	99.0	+	12	0.85	0.0360*
0.63	1373	96.5	+	18	1.31	0.0016**
1.25	1466	103.2	+	21	1.43	0.0006**
2.5	1347	93.2	+	20	1.48	0.0005**
5	1501	105.8	+	25	1.67	0.0001**
10	1447	101.8	+	32	2.21	<0.0001**
PC	1347	95.2	+	31	2.30	<0.0001**

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)

6.3.2.3 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. The results evaluated by BioReliance are shown in Table 17. The MTF values of the test chemical doses ranged from 2.92 to 5.52%. All test chemical concentrations induced a significant increases in MTF compared to the VC ($p < 0.05$).

Table 17: 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%)	1372	100	+	6	0.44	-
0.25	1335	97.3	+	39	2.92	<0.0001**
0.5	1254	92.2	+	52	4.15	<0.0001**
1	1242	90.9	+	44	3.54	<0.0001**
2.5	1159	85.7	+	64	5.52	<0.0001**
5	1143	83.7	+	43	3.76	<0.0001**
10	1010	75.4	+	50	4.95	<0.0001**
PC	1207	88.3	+	24	1.99	0.0002**

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 Concurrent cytotoxicity (Relative Plating Efficiency)

Figure 14 shows the results of the RPE test from all laboratories. 3-Methylcholanthrene was not very cytotoxic at the doses tested at BASF and Harlan CCR, although at BioReliance the top dose of 10 µg/ml produced a 25% toxicity.

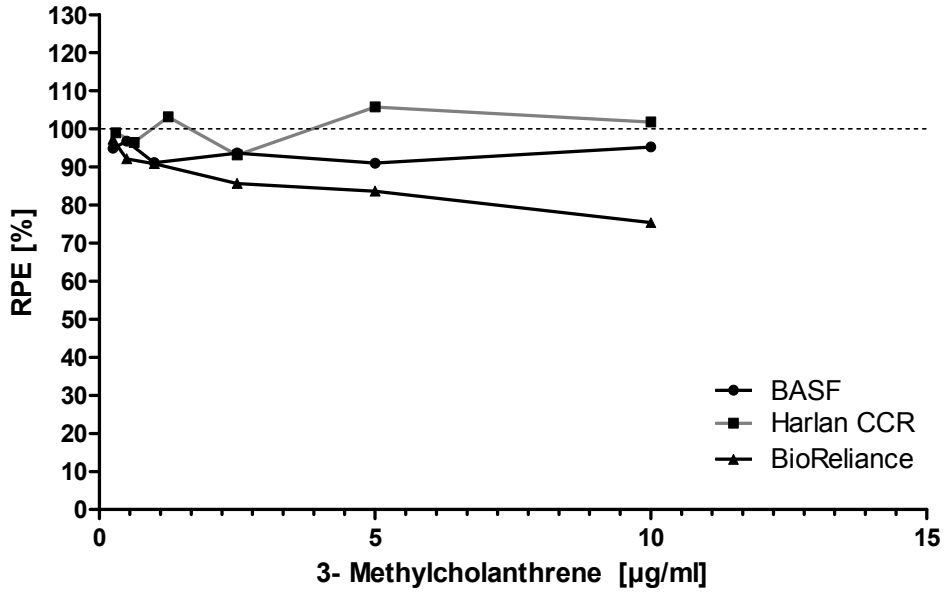

Figure 14: Relative plating efficiency (RPE) compared to vehicle control in all laboratories testing coded 3-methylcholanthrene

6.3.2.5 Morphological transformation frequency

MTF results with 3-methylcholanthrene are shown in Figure 15. Despite the differences in cytotoxicity, all laboratories showed 3-methylcholanthrene to induce a statistically significant increase in morphological transformation at all doses tested, with a clear dose-related response for Harlan CCR. The reason for the higher MTF observed by BioReliance is not clear.

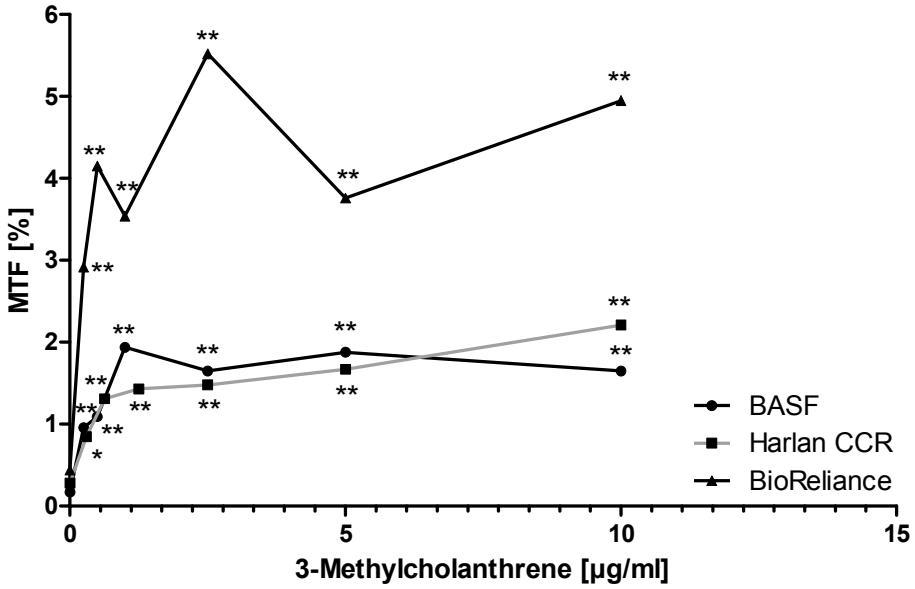

Figure 15: Morphological Transformation Frequency (MTF) shown as a percentage of transformed colonies compared to total colonies for all laboratories testing coded 3-methylcholanthrene

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

6.3.2.6 Acceptance criteria and assessment

All acceptance criteria were met in all laboratories. 3-Methylcholanthrene was assessed to be positive by all laboratories (Table 18).

Table 18: Acceptance criteria and assessment of 3-methylcholanthrene results

3-Methylcholanthrene			
Criteria	**Laboratory**		
	BASF	**Harlan CCR**	**BioReliance**
Number of scorable colonies per treatment group ≥1000 Or <1000 if positive result	**yes** (≥1593)	**yes** (≥1347)	**yes** (≥1010)
Average number of 25-45 colonies per dish or >45 colonies if positive result or <25 colonies if negative result	**yes** (41.3-45.3)	**yes** (33.2-37.7)	**yes** (26.0-34.5)
Plating Efficiency of vehicle control >20%	**yes** (41.2%)	**yes** (41.9%)	**yes** (28.8%)
Morphological Transformation Frequency of vehicle control <0.6%	**yes** (0.17%)	**yes** (0.28%)	**yes** (0.44%)
Number of scorable concentrations ≥5	**yes** (6)	**yes** (6)	**yes** (6)
Fisher's test *p*-value of positive control <0.05	**yes** (<0.0001)	**yes** (<0.0001)	**yes** (0.0002)
Fulfilment of all assay acceptance criteria	**YES**	**YES**	**YES**
Assessment	+	+	+

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 6.7 CTA. These results agree with published data (LeBoeuf *et al*., 1989; Dusinska and Slamenova, 1994).

6.4 o-Toluidine HCl

6.4.1 Dose-Range finding test

Figure 16 shows the results of the DRF tests with o-toluidine HCl. Harlan CCR results did not show signs of cytotoxicity at the concentrations tested whereas BioReliance results show a cytotoxicity up to IC_{10}. BASF did not perform DRFs because a dose range was suggested by the VMT and was considered by this laboratory adequate for choosing the doses.

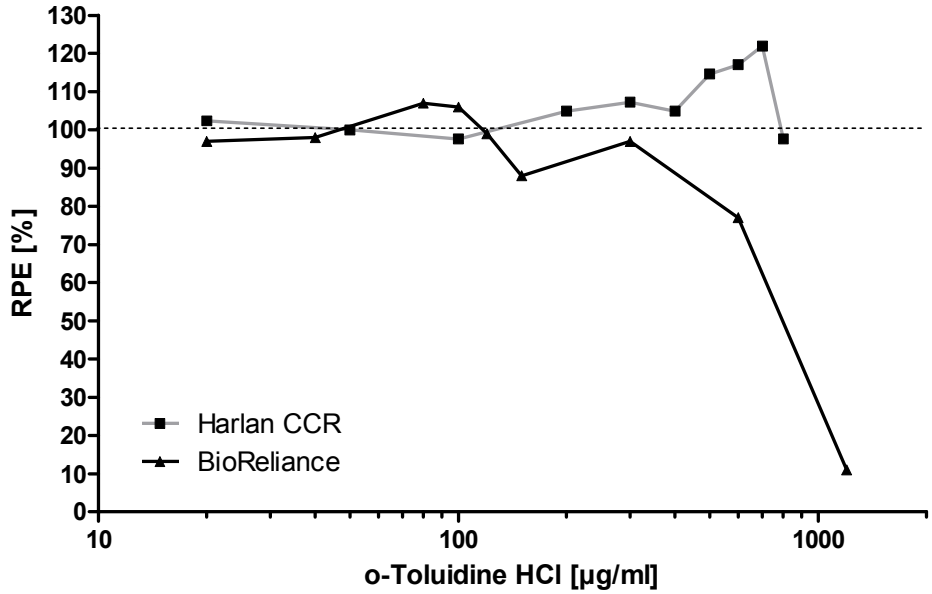

Figure 16: Relative Plating Efficiency (RPE) compared to vehicle control in all laboratories testing coded o-toluidine HCl, for Dose-Range Finding

6.4.2 Transformation assay

VCs gave transformation frequencies within the expected range (≤0.6%): 0.36% (BASF), 0.13% (Harlan CCR), 0.34% and 0.29% (BioReliance). The PC chemical benzo(a)pyrene led to the expected increase in morphologically transformed colonies: 2.21% (BASF), 1.84% (Harlan CCR), 1.54% and 1.34 % (BioReliance).

6.4.2.1 BASF

o-Toluidine HCl was dissolved in DMEM-L. The concentrations were selected and evaluated based on the suggested dose range from the VMT (Table 19). 1200 µg/ml was selected as the top dose however the cytotoxicity was so high that no colonies formed at that dose. The MTF values of the test chemical doses ranged from 0.78 to 1.76%. All concentrations induced significant increases in MTF compared to the VC ($p < 0.01$), except the lowest and the highest concentrations.

Table 19: 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 (DMEM-L)	1402	100.0	+	5	0.36	-
20	1495	112.2	+	13	0.87	0.0628
40	1529	112.3	+	17	1.11	0.014*
60	1609	115.0	+	23	1.43	0.0015**
150	1593	119.8	+	28	1.76	0.0001**
300	1582	115.9	+	23	1.45	0.0013**
600	1150	86.2	++	9	0.78	0.1192
PC	1541	110.1	+	34	2.21	<0.0001**

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)

6.4.2.2 Harlan CCR

o-Toluidine HCl was dissolved in DMSO. The concentrations were selected on the basis of the suggested dose range from the VMT and the DRF test. The results evaluated by Harlan CCR are shown in Table 20. The MTF values of the test chemical doses ranged from 0.87 to 2.66%. All concentrations induced significant increases in MTF compared to the VC ($p < 0.01$), with a dose-dependent effect.

Table 20: 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%)	1535	100	+	2	0.13	-
300	1618	105.7	+	14	0.87	0.0028**
400	1698	110.7	+	19	1.12	0.0002**
500	1643	107.7	+	26	1.58	<0.0001**
600	1610	104	+	26	1.61	<0.0001**
700	1422	93.5	++	30	2.11	<0.0001**
800	1316	85.8	++	35	2.66	<0.0001**
PC	1574	102.6	+	29	1.84	<0.0001**

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

6.4.2.3 BioReliance

o-Toluidine HCl was dissolved in DMSO (TA1). The concentrations were selected and evaluated based on the suggested dose range from the VMT and the DRF test (Table 21). 1200, 1000 and 800 µg/ml were also selected, however the cytotoxicity was so high that no colonies formed at those concentrations. Different colony size and density than those in previous experiments were observed at all concentrations.

Table 21: Transformation assay TA1 results from BioReliance, testing coded o-toluidine HCl

BioReliance o-toluidine HCl (µg/ml) TA1	Scorable colonies	RPE (%)	Colony density/size	Transformed colonies	MTF (%)	Fisher's exact test *p*-value (one-sided)
VC (DMSO 0.2%)	1771	100	+	6	0.34	-
100	1329	75.2	++	12	0.90	0.0359*
200	1427	80.7	++	7	0.49	0.3456
300	1464	82.7	++	3	0.20	0.8544
400	1413	79.9	++	7	0.50	0.3389
500	1567	88.6	++	4	0.26	0.7738
600	1646	92.9	++	5	0.30	0.6832
PC	1816	102.5	+	28	1.54	0.0001**

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)

Only one concentration (100 µg/ml) induced a statistically significant increase in MTF (without a trend). As such, the results were considered to be inconclusive. Therefore, the VMT (during the meeting in May 2007) recommended that BioReliance repeated the experiment using a narrower concentration range around 100 µg/ml, which was the dose that gave a statistically significant increase in the number of transformed colonies.

Table 22 reports the results from the second experiment TA2. The MTF values of the test chemical doses ranged from 0.73 to 1.49%. All concentrations, except the lowest one, induced significant increases in MTF compared to the VC ($p < 0.05$).

Table 22: Transformation assay TA2 results from BioReliance, testing coded o-toluidine HCl using a narrower concentration range

BioReliance o-toluidine HCl (µg/ml) TA2	Scorable colonies	RPE (%)	Colony density/size	Transformed colonies	MTF (%)	Fisher's exact test *p*-value (one-sided)
VC (DMSO 0.2%)	1359	100.0	+	4	0.29	-
20	1364	101.0	+	10	0.73	0.0903
40	1276	94.2	+	12	0.94	0.0286*
80	1178	89.6	+	17	1.44	0.0013**
100	1070	81.0	+	12	1.12	0.0120*
120	1094	84.3	+	19	1.74	0.0002**
150	1275	97.9	+	19	1.49	0.0008**
PC	1196	90.1	+	16	1.34	0.0025**

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)

6.4.2.4 Concurrent cytotoxicity (Relative Plating Efficiency)

Cytotoxicity of o-toluidine HCl was evaluated by RPE assessment (Figure 17). o-Toluidine HCl showed limited cytotoxicity in all laboratories. Only a very limited number of doses were cytotoxic for BASF and Harlan CCR, while the shape of the BioReliance curves was different from that of the others.

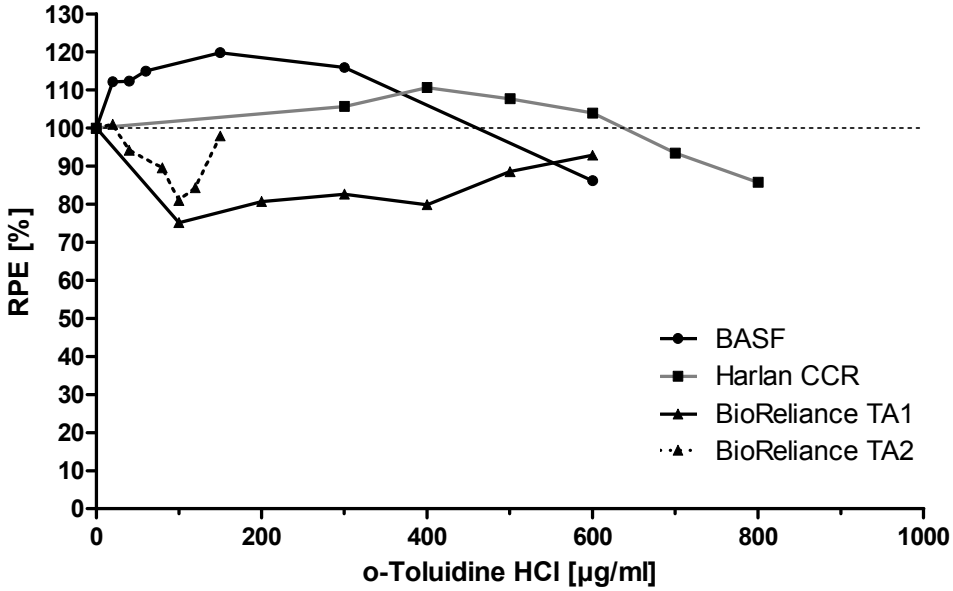

Figure 17: Relative plating efficiency (RPE) compared to vehicle control in all laboratories testing coded o-toluidine HCl

6.4.2.5 Morphological transformation frequency

MTF results with o-toluidine HCl are shown in Figure 18. o-Toluidine HCl induced a statistically significant increase in morphological transformation in all laboratories, with a clear dose-dependent response for Harlan CCR.

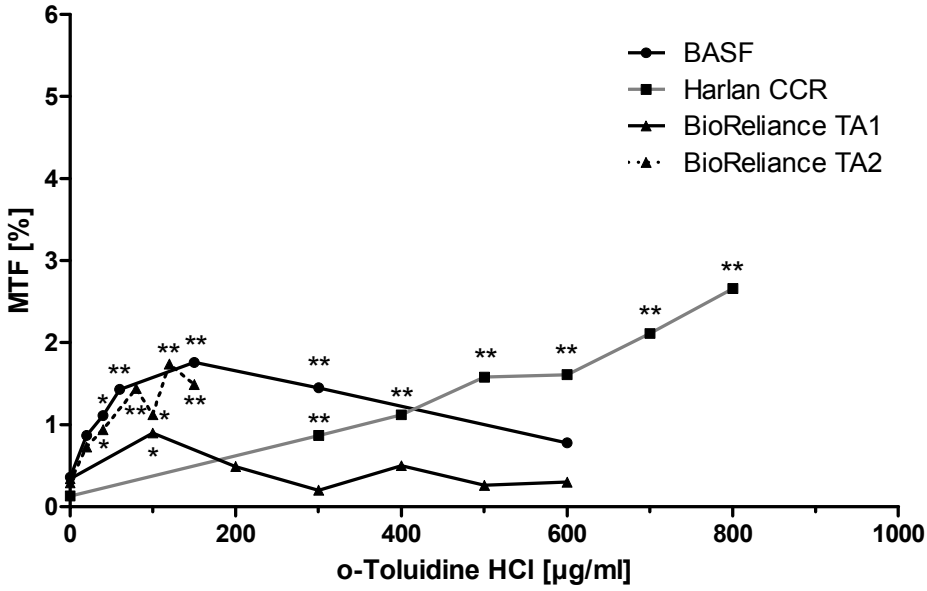

Figure 18: Morphological Transformation Frequency (MTF) shown as a percentage of transformed colonies compared to total colonies, for all laboratories testing o-Toluidine HCl

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

6.4.2.6 Acceptance criteria and assessment

The acceptance criteria were fulfilled by all laboratories. o-Toluidine HCl was assessed to be positive by all laboratories (Table 23).

Table 23: Acceptance criteria and assessment of o-toluidine HCl results

o-Toluidine HCl				
Criteria	**Laboratory**			
	BASF	**Harlan CCR**	**BioReliance (TA1)**	**BioReliance (TA2)**
Number of scorable colonies per treatment group ≥1000 or <1000 if positive result	**yes** (≥1150)	**yes** (≥1316)	**yes** (≥1329)	**yes** (≥1070)
Average number of 25-45 colonies per dish or >45 colonies if positive result or <25 colonies if negative result	**yes** (30.6-42.5)	**yes** (33.1-42.6)	**yes** (33.4-45.5[#])	**yes** (27.6-34.4)
Plating Efficiency of vehicle control >20%	**yes** (32.2%)	**yes** (45.3%)	**yes** (37.0%)	**yes** (28.4%)
Morphological Transformation Frequency of vehicle control <0.6%	**yes** (0.36%)	**yes** (0.13%)	**yes** (0.34%)	**yes** (0.29%)
Number of scorable concentrations ≥5	**yes** (6)	**yes** (6)	**yes** (6)	**yes** (6)
Fisher's test *p*-value of positive control <0.05	**yes** (<0.0001)	**yes** (<0.0001)	**yes** (<0.0001)	**yes** (0.0025)
Fulfilment of all assay acceptance criteria	**YES**	**YES**	**YES**	**YES**
Assessment	+	+	**i**[##]	+

[#] 45.5 colonies per dish for the positive control

[##] i = inconclusive, since only one concentration (100 µg/ml) induced a statistically significant increase in MTF (without a trend)

6.4.3 Conclusion

Treatment with o-toluidine HCl produced a statistically significant increase in morphologically transformed colonies in all laboratories. One of the laboratories initially produced an inconclusive result, which was confirmed to be positive upon repetition of the experiment. As such, o-toluidine HCl was considered to be a positive transforming agent in the SHE pH 6.7 CTA. These results agree with published data (Kerckaert *et al*., 1998; Engelhardt *et al*., 2004).

6.5 Phthalic anhydride

6.5.1 Dose-range finding test

Figure 19 shows the results of the DRF tests with phthalic anhydride. The results from BASF showed limited cytotoxicity, whereas Harlan CCR and BioReliance results showed a cytotoxicity up to IC_{25} - IC_{10} since they tested to higher concentrations than BASF.

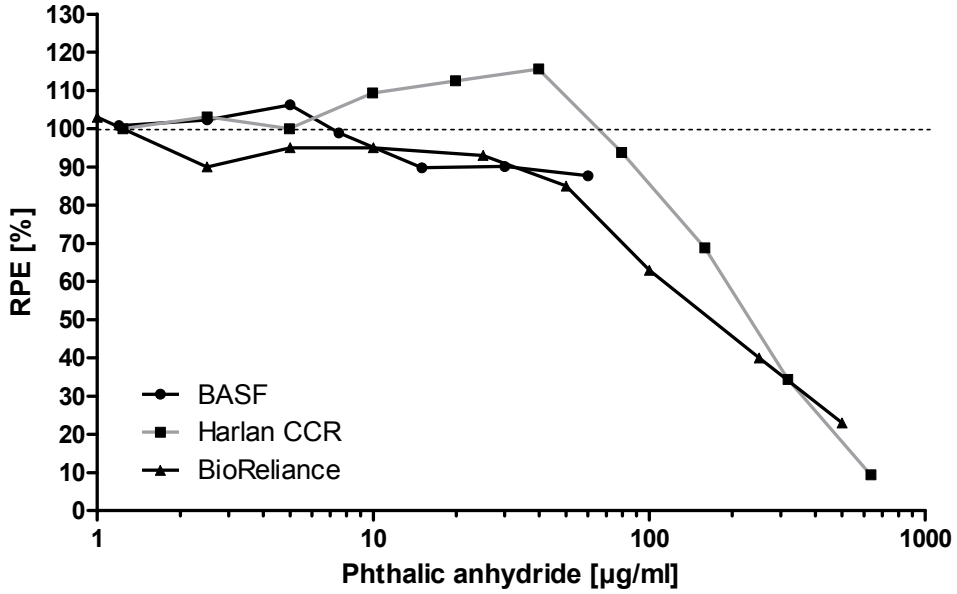

Figure 19: Relative plating efficiency (RPE) compared to vehicle control in all laboratories testing coded phthalic anhydride, for Dose-Range Finding

6.5.2 Transformation assay

VCs gave transformation frequencies within the expected range (≤0.6%): 0.23% (BASF), 0.13% (Harlan CCR) and 0.45% (BioReliance). The PC chemical benzo(a)pyrene led to the expected increase in morphologically transformed colonies: 1.71% (BASF), 1.46% (Harlan CCR) and 2.21% (BioReliance).

6.5.2.1 BASF

Phthalic anhydride was dissolved in DMSO. Phthalic anhydride precipitated in the pre-mixture with medium (double concentration) at 60 µg/ml, therefore this was chosen as the top concentration. The concentrations were selected on the basis of the DRF test and the results evaluated by BASF are shown in Table 24. The MTF values of the test chemical doses ranged from 0.24 to 1.50%. The two highest test chemical concentrations induced significant increases in MTF compared to the VC ($p < 0.05$), with a dose-dependent effect.

The mean number of colonies in the VC was 45.3 (slightly above the limit in the acceptance criteria of 45). However, this assay was still considered valid by the VMT since the difference was minimal and a clear dose range increase of the MTF was observed.

BASF confirmed those results in a second experiment (data not shown) focusing on the concentrations of interest and showed a significant dose-related increase in MTF at three consecutive doses (30, 60, 90 µg/ml).

Table 24: 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%)	1703	100.0	+	4	0.23	-
1.2	1233	76.8	+	3	0.24	0.623
2.5	1189	74.4	+	4	0.34	0.4318
5	1338	80.5	+	5	0.37	0.3549
7.5	1196	75.1	+	5	0.42	0.2931
15	1180	76.6	+	5	0.42	0.286
30	1038	68.4	+	8	0.77	0.041*
60	1198	76.6	+	18	1.50	0.0001**
PC	1405	83.7	+	24	1.71	<0.0001**

[#] = average number of colonies > 45 (45.3)
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)

6.5.2.2 Harlan CCR

Phthalic anhydride was dissolved in DMSO. Phthalic anhydride precipitated in the pre-mixture with medium (double concentration) at 318 µg/ml, therefore this was chosen as the top concentration. The concentrations were selected on the basis of the DRF test and the results evaluated by Harlan CCR are shown in Table 25. The MTF values of the test chemical doses ranged from 0.70 to 3.15%. All concentrations induced significant increases in MTF compared to the VC ($p < 0.05$), with a dose-related response although the highest concentrations were highly cytotoxic.

Table 25: 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%)	1505	100.0	+	2	0.13	-
32	1673	111.4	+	12	0.72	0.0108*
80	1580	105.3	+	11	0.70	0.0139*
160	1479	76.0	++	37	2.50	<0.0001**
200	1900	63.2	++	45	2.37	<0.0001**
240	1809	50.2	++	57	3.15	<0.0001**
280	1333	31.7	+++	40	3.00	<0.0001**
320	1041	21.6	+++	23	2.21	<0.0001**
PC	1640	109.5	+	24	1.46	<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)

6.5.2.3 BioReliance

Phthalic anhydride was dissolved in DMSO. The concentrations were selected on the basis of the DRF test. The results evaluated by BioReliance are shown in Table 26. The MTF values of the test chemical doses ranged from 0.56 to 1.45%. Only the two highest concentrations induced significant increases in MTF compared to the VC ($p < 0.05$).

Table 26: 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%)	1348	100.0	+	6	0.45	-
5	1389	103.1	+	12	0.86	0.1314
10	1425	105.7	+	8	0.56	0.4363
25	1197	88.8	+	12	1.00	0.0750
50	1125	83.4	+	16	1.42	0.0088**
100	1239	61.4	+	18	1.45	0.0063**
250	-	-	++	-	-	-
PC	1445	107.5	+	32	2.21	<0.0001**

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

6.5.2.4 Concurrent cytotoxicity (Relative Plating Efficiency)

Cytotoxicity of phthalic anhydride was evaluated by RPE assessment (Figure 20). Phthalic anhydride was shown to be cytotoxic in all laboratories, with a clear dose-dependent effect for Harlan CCR and Bioreliance. However, it is not clear why the cytotoxicity curves look different in the different laboratories.

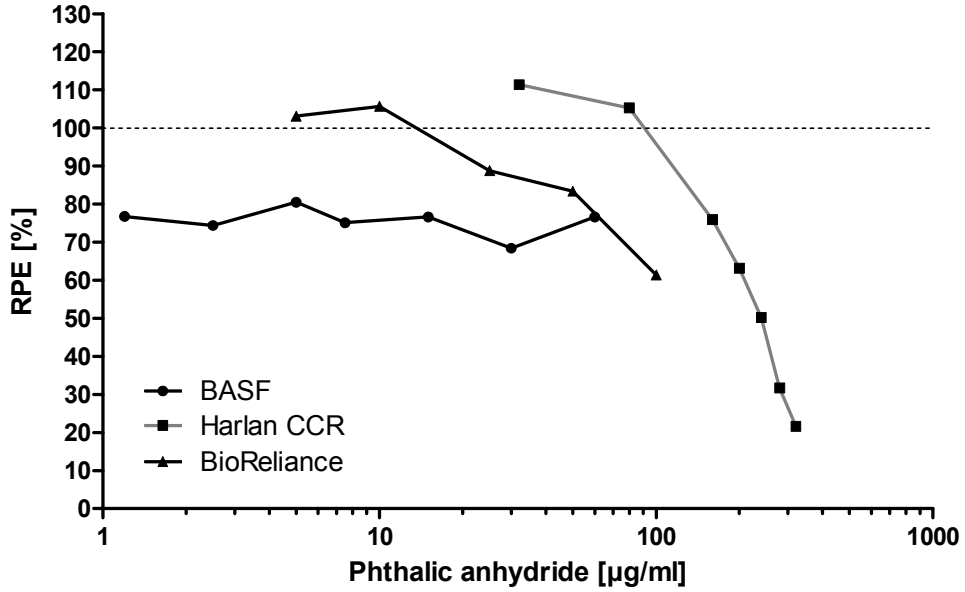

Figure 20: Relative plating efficiency (RPE) compared to vehicle control in all laboratories testing coded phthalic anhydride

6.5.2.5 Morphological transformation frequency

MTF results with phthalic anhydride are shown in Figure 21. Phthalic anhydride induced a statistically significant, dose-dependent increase in morphological transformation at concentrations above 32 µg/ml.

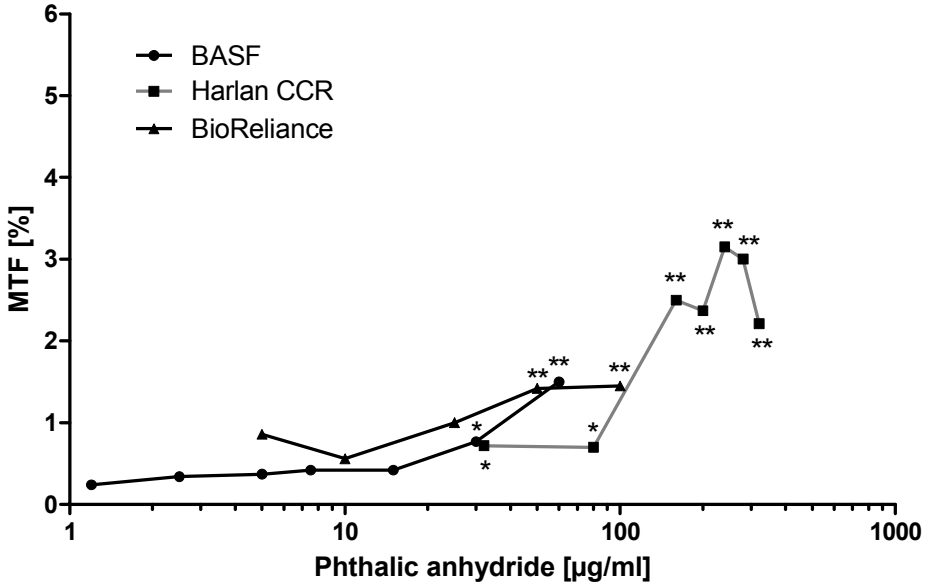

Figure 21: Morphological Transformation Frequency (MTF) shown as a percentage of transformed colonies compared to total colonies for all laboratories testing coded phthalic anhydride

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

6.5.2.6 Acceptance criteria and assessment

For Harlan CCR and BioReliance, all acceptance criteria were fulfilled. For BASF, all acceptance criteria were met except that the mean number of colonies in the VC was slightly above the limit of 45 (45.3). However, the VMT considered this test acceptable for the overall evaluation of the study, since the difference was minimal and a clear dose range increase of the MTF was observed. Phthalic anhydride was assessed to be positive by all laboratories (Table 27).

Table 27: Acceptance criteria and assessment of phthalic anhydride results

Phthalic anhydride			
Criteria	**Laboratory**		
	BASF	**Harlan CCR**	**BioReliance**
Number of scorable colonies per treatment group≥1000 or <1000 if positive result	**yes** (≥1038)	**yes** (≥1041)	**yes** (≥1125)
Average number of 25-45 colonies per dish or >45 colonies if positive result or <25 colonies if negative result	**no**[#] (31.0-45.3)	**yes** (26.1-47.6)	**yes** (28.1-36.3)
Plating Efficiency of vehicle control >20%	**yes** (41.2%)	**yes** (37.7%)	**yes** (28.1%)
Morphological Transformation Frequency of vehicle control <0.6%	**yes** (0.23%)	**yes** (0.13%)	**yes** (0.45%)
Number of scorable concentrations ≥5	**yes** (7)	**yes** (7)	**yes** (5)
Fisher's test *p*-value of positive control <0.05	**yes** (<0.0001)	**yes** (<0.0001)	**yes** (<0.0001)
Fulfilment of all assay acceptance criteria	**NO**[##]	**YES**	**YES**
Assessment	+	+	+

[#] 45.3 colonies per dish for vehicle control
[##] considered acceptable for overall study evaluation despite a number of colonies for vehicle control very slightly beyond limit (>45)

6.5.3 Conclusion

Treatment with phthalic anhydride produced a reproducible statistically significant increase in morphologically transformed colonies in all laboratories. As such, under the conditions of the test as performed, phthalic anhydride was considered to be a positive transforming agent in the SHE pH 6.7 CTA.

These results are contradictory to published data (LeBoeuf *et al*., 1996; NTP), in which phthalic anhydride is classified as a non-carcinogen and was found to be negative in both mice and rat bioassays.

6.5.4 Analysis of phthalic anhydride by mass spectrometry

It was decided to verify that the chemical shipped to and used by the laboratories was indeed phthalic anhydride and that no mistake had been made during the chemical distribution. To do so, coded phthalic anhydride samples were sent to ECVAM from J&JPRD and BioReliance for chemical analysis. The samples (Fluka product no. 80018, Lot no. 1173489) were analysed by mass spectroscopy and compared to a new lot of phthalic anhydride (Fluka product no. 80018, Lot no. 1339610) and to phthalic anhydride from another company (Sigma product no. 320064-25G, Lot no. 11908MC276).

The results of the analysis confirmed that all the coded samples returned to ECVAM were indeed phthalic anhydride (Annex 12.4). Phthalic anhydride was also tested for SHE cell transformation by University of Metz at this same time, and later by the other laboratories using the SHE pH 7.0 protocol and was found to be negative (see report on Prevalidation of SHE pH 7.0 CTA). The chemical used by University of Metz was also analysed by mass spectrometry at ECVAM and showed exactly the same profile as phthalic anhydride used by the other laboratories.

Overall, the chemical identification analysis demonstrated that phthalic anhydride was used by all of the laboratories in the present study, as expected.

6.6 Overview on vehicle and positive controls

All control values reported in the experiments described in the above sections were pooled together in Figure 22. The response of the VC is very similar between laboratories and it is within the acceptable limits, *i.e.* MTF below 0.6%. Although the response of the PCs appears more scattered, all PCs are above the acceptable limit, *i.e.* MTF above 0.6%.

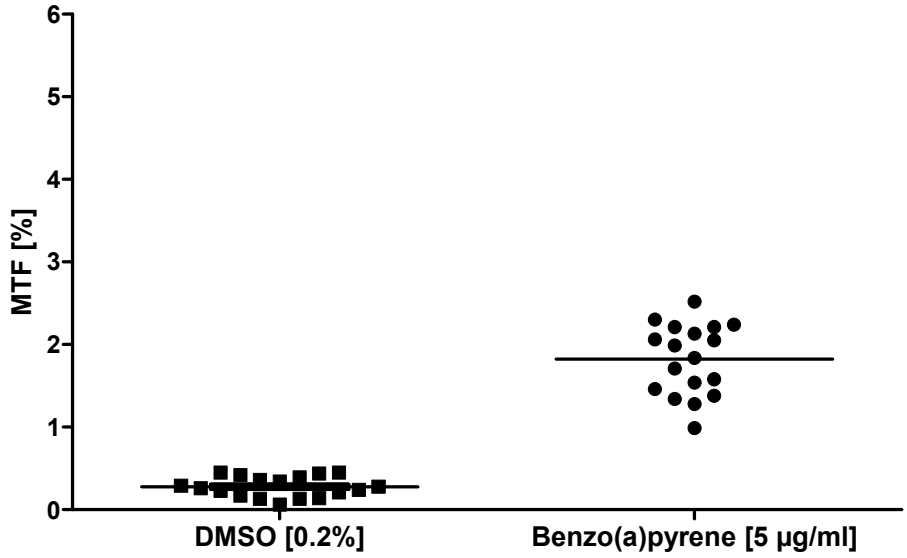

Figure 22: Morphological Transformation Frequency (MTF) of vehicle (■) and positive (●) controls for all cell transformation experiments, in all laboratories

6.7 Conclusion of the Validation Management Team on Module 4

The between-laboratory reproducibility was shown to be good. All chemicals tested as well as vehicle and positive controls gave reproducible results in the three laboratories.

It should be noted that, 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 the vehicle choice in the CTA.

7 SUMMARY OF RESULTS

An optimised and standardised protocol was evaluated in this 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. Using the standardised protocol, all laboratories showed good reproducibility within as well as between laboratories. Table 28 summarises of the results obtained with each chemical.

Table 28: Summary table of between-laboratory reproducibility results

Chemical	Expected result[#]	Laboratory		
		BASF	Harlan CCR	BioReliance
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 of 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 also showed both a good within- and between-laboratory reproducibility.

7.2 Anthracene

The results of 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 of 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 of 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 of 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 final results of phthalic anhydride were reproducible between the laboratories and induced a dose responsive, statistically significant increase in MTF. However phthalic anhydride was shown to be positive in the CTA, which is in conflict with data from the literature (NTP database, http://www.ntp-server.niehs.nih.gov). To ascertain if the laboratories had really tested phthalic anhydride, the remaining samples used by all laboratories were returned to ECVAM and were analysed by mass spectroscopy. These samples were also compared to other lots of the same chemical obtained from Fluka and Sigma. The results showed that all samples were the same and that the chemical tested in the CTA was indeed phthalic anhydride.

This difference could be due to pH dependent instability of phthalic anhydride in the aqueous media used in the SHE assay. According to the OECD Screening Information Data Set (SIDS) document on phthalic anhydride (April 2005), phthalic anhydride is unstable in water, hydrolyzing within minutes completely to phthalic acid which is non-genotoxic. Importantly, experiments with phthalic anhydride performed in the presence of buffer showed a half-life for phthalic anhydride of 30.5 seconds at pH 7.24 and at pH 6.8 the half-life of phthalic anhydride in water was prolonged to 61 seconds. Thus, small differences in the dose preparation, timing and pH stability of phthalic anhydride in the SHE assays could have contributed to the conflicting results obtained in this study.

8 OVERALL CONCLUSION BY THE VALIDATION MANAGEMENT TEAM

The aim of the study was to prevalidate the SHE CTA at pH 6.7, 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 29 summarises the conclusions made by the VMT on the assessment of the SHE pH 6.7 CTA.

Table 29: 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 chemical	**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 6.7 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 a similar protocol as reported in the literature (LeBoeuf *et al*., 1989; Engelhardt *et al*., 2004) 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 from this effort, points that should 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. 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. The photo catalogue produced during this study has proven to be an invaluable aid in establishing consistency in assessing colony morphology and for scoring of the experiments performed to assess the between-laboratory reproducibility. 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.

- In the course of the study it was realised that results can be inconclusive. Therefore, it is recommended that 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 test chemical concentrations that fulfil the assay acceptance criteria should be available to conclude on 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.

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12 ANNEXES

12.1 Chemicals selected for the prevalidation of SHE pH 6.7 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 30.

Table 30: Genotoxicity and carcinogenicity data on the chemicals selected for the SHE pH 6.7 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 6.7 Cell Transformation Assay protocol

PROTOCOL

The low pH 6.7 Cell Transformation Assay in

Syrian Hamster Embryo Cells

(SHE Assay)

November 2005

(Amended May 2006)

Prepared by BASF Aktiengesellschaft

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5. REFERENCES

1. GENERAL REQUIREMENTS

1.1. AIM OF THE STUDY / GUIDELINES

Study of morphological transformation *in vitro* in SHE cells in agreement with a published test protocol (1) taking into consideration recent modifications (2) and in accordance with the following guideline:

- OECD draft proposal (1996): *In vitro* Syrian Hamster Embryo (SHE) Cell Transformation Assay, 1996

1.2. BACKGROUND OF THE CELL TRANSFORMATION ASSAY (CTA)/SHE

Syrian hamster embryo (SHE) cell cultures have been used for testing chemicals for malignant transformation of mammalian cells since Berwarld and Sachs (3) introduced this *in vitro* system.

SHE cells are derived from embryos of pregnant (13 - 13.5 days gestation) Syrian golden hamsters. SHE cells are diploid and genetically stable primary cells. The cell population comprises of multiple cell type and cells at various stages in the differentiation process 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 (20 - 40%) and low spontaneous transformation frequency.

In contrast to most *in vitro* assays that measure the capacity of a compound to induce a very specific event, such as mutations or chromosomal aberrations, the SHE assay uses a transformed phenotype as the endpoint. Since cancer is recognized as a multi-step process, with many possible mechanisms leading to the ultimate transforming event, the use of a more general endpoint, such as the transformation of cells *in vitro*, may be of high predictive value. LeBoeuf *et al.* (4) have demonstrated that SHE cells, when cultured in an environment of reduced bicarbonate (low pH), will be morphologically transformed at a rate high enough to allow for rigorous statistical evaluation.

The CTA using SHE cells consists in the estimation of the concurrent cytotoxicity and morphological transformation. The duration of the test is 9 days in total.

The endpoint of the cytotoxicity is the formation of colonies measured as Plating Efficiency (PE) and the endpoint of the neoplastic potential is the presence of morphologically transformed colonies.

Concentrations to be used for the CTA are previously determined by a dose-effect curve to assess the cytotoxicity of the chemical.

1.3. LABORATORY DATA TO BE STORED

All laboratory and measured data generated during the conduct of the study will be recorded and retained in an appropriately labelled file in the laboratory until the draft report is completed as indicated in the Statement of Work.

1.4. RETENTION OF RECORDS

At the end of the pre-validation / validation study, copies of the original raw and derived assay data, as well as copies of other raw data to these studies (instrument logs, calibration records, facility logs, etc.), shall be submitted to ECVAM for storing and archiving (under the direction of the Management Team).

All relevant documents and materials will be retained at the laboratory at least five years after completion of the studies.

The petri dishes of all experiments must be stored and archived at the participating testing facilities until the end of the pre-validation study.

1.5. STUDY CONDUCT IN THE SPIRIT OF GLP

The study will be carried out in the spirit of GLP.

The investigations described below will be carried out in accordance with the Standard Operating Procedures (SOPs) unless reasons for using a different procedure have been given and are documented in amendments to this Protocol.

All deviations from the SOPs and this Protocol will be documented immediately in the raw data and have to be countersigned by the Study Director.

2. MATERIAL AND METHODS

2.1. STERILITY OF THE TEST SYSTEM

All cell culture applications shall be conducted under aseptic conditions. The presence of bacterial or fungal contamination in the cultures shall be determined by gross visual inspection during and at the conclusion of each assay. If bacterial or fungal contamination is present in the cultures, the Laboratory Director shall determine the course of action.

2.2. CULTURE MEDIA AND REAGENTS

All working media will be supplied by certified sponsors. All media will be labelled with batch/lot no, date of preparation, storage condition and expiration date.

- **Wash solution for cell isolation**

HBSS-CMF (Hank´s balanced salt solution; calcium/magnesium free) containing 200 U/ml of penicillin and 200 µg/ml streptomycin.
The stability period is 4 weeks at 2 - 8°C.

- **Dissociation solution for SHE cell isolation**

HBSS-CMF (Hank´s balanced salt solution; calcium/magnesium free) containing 200 U/ml penicillin, 200 µg/ml streptomycin, 1.25% Enzar - T trypsin and 2.5% pancreatin.

The pH of the dissociation solution should be neutral (~7.0)
(correction of the pH with 7.5% sodium bicarbonate solution).

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

- **Cell isolation medium**

The cell isolation medium consists of the culture medium DMEM-L (Dulbecco's Modified Eagle's Medium - LeBoeuf's modification) containing 20% foetal bovine serum, 200 U/ml of penicillin, 200 µg/ml streptomycin and 2 mM L-glutamine.

The pH of the cell isolation medium should be neutral (~7.0) and after incubation at 37 ± 1° with 10 ± 0.5% CO_2 in a humidified incubator for at least four hours should be between 6.65 and 6.75 (correction of the pH with 7.5% sodium carbonate solution).

The stability period is 4 weeks at 2 - 8 °C.

- **Complete culture medium**

The complete culture medium consists of the culture medium DMEM-L (Dulbecco's Modified Eagle's Medium - LeBoeuf's modification) containing 20% foetal bovine serum and 2 mM L-glutamine.

The pH of the complete culture medium should be neutral (~7.0) and after incubation at 37 ± 1°C with 10 ± 0.5% CO_2 in a humidified incubator for at least four hours should be between 6.65 and 6.75 (correction of the pH with 7.5% sodium bicarbonate solution).

The stability period is 4 weeks at 2 - 8°C.

- **Cryopreservation medium**

To deep-freeze the cells, the complete culture medium is supplemented with15% DMSO

- **SHE cell detachment solution**

HBSS-CMF (Hank's balanced salt solution; calcium/magnesium free) containing 0.05% trypsin and 0.02% Na2EDTA or with the addition of 0.25% trypsin in a ratio of 1:1.

- **Other solutions and reagents**

– Foetal bovine serum: the serum should be tested prior to the experiments and along with DMSO and benzo(a)pyrene to ensure that it meets the following criteria:
  - the number of colonies in the vehicle control (DMSO) should be between 25 and 45,
  - colonies should be normal sized,
  - positive control should significantly induce morphologically transformed colonies and within the historical value range of the laboratory for positive controls,
  - vehicle control should lead to morphological transformation within the historical value range of the laboratory for vehicle controls.

– HBSS-CMF (Hank's balanced salt solution; calcium/magnesium free)

– 0.4% trypan blue

2.3. EXPERIMENTAL DESIGN

The experimental design includes a first step where a dose-response curve is generated to assess the cytotoxicity of each compound. On the basis of the cytotoxicity curve, the doses for the morphological transformation will be chosen. During the second step, cytotoxicity and morphological transformation of the selected doses will be performed in parallel.

Cell cultures used for cytotoxicity and transformation experiments were established with both SHE feeder cells (irradiated with X-rays sufficient to prevent cell division) and a sufficient number of target SHE cells (not irradiated) to produce about 25 - 45 colonies per ø60 mm-culture dish, with an optimum of 35 colonies/dish.

2.3.1. Choice of the vehicle

DMSO or medium (complete culture medium) should be selected as the vehicle.
The final concentration of DMSO in the dishes should not exceed 0.2%.

2.3.2. Preliminary Cytotoxicity Assay

The testing procedure for the preliminary cytotoxicity assay is similar to that described in the main experiment with the following exceptions:

- Number of plates seeded per dose is 10.

- The number of target cells seeded is the same for all the doses.

The maximum dose will be determined on a case-by-case basis taking into account solubility and any relevant cytotoxicity information available for the test article. A range of doses (at least 10) will be applied to achieve a wide toxicity range. The highest dose level tested for soluble test articles will be 5 mg/ml or 10 mM, whichever is lower. Test article dosing preparations will be prepared fresh on the same day as the cultures are dosed.

2.4. MAIN EXPERIMENT

2.4.1. Dose selection for the main experiment

The relative cytotoxicity of each treatment group from the dose range-finding study 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.

These 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 morphological cell transformation (MT), definitive assay doses should include, if possible:

- a high dose causing at least 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,

If the test article is essentially nontoxic, then at least five concentrations will be selected up to a maximum of 5 mg/ml or 10 mM (whichever is lower), solubility permitting. For nontoxic and insoluble test articles, the highest dose level tested will be within 2-times the visible solubility limit in complete medium. For toxic and insoluble test articles, 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.

2.4.2. Adjusted target cells seeding

For routine assay performance, an average range of 25 - 45 analyzable colonies per dish has been recommended (1, 2). To achieve this, the number of target cells seeded in the main experiment must be adjusted accordingly (increased) for test substance doses that are toxic or are expected to be toxic.

The cell number should be adjusted: Approximately 30% reduction: 1.5x adjustment; approximately 50% reduction: 2x adjustment.

2.4.3. Control articles

- **Vehicle control**

The vehicle controls will contain the vehicle selected for the test substance at the same concentration and volume used in the test cultures.

- **Positive controls**

Five µg benzo[a]pyrene (B[a]P)/ml of culture medium will be tested to demonstrate the sensitivity of the test method. The positive control will be dissolved in DMSO.

2.4.4. Cell Transformation Assay

A sufficient number of SHE cells 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 about 4 x 10^4 SHE feeder cells in 2 ml of complete medium.

The transformation assay may be a single trial that results in at least 1 000 colonies (40 dishes) per treatment group to establish a negative result. The assay will include at least five scorable concentrations of test compound and the appropriate vehicle and positive controls. The dosed cell cultures will be incubated for a period of 7 days following treatment initiation. The culture dishes will be labelled with the assay number, trial number, and dose code.

After the incubation period, the culture dishes in each treatment group may be washed with Hank's balanced salt solution (HBSS), fixed with methanol, and stained with 10 - 15% aqueous Giemsa stain. After rinsing with tap water, the dishes will be air-dried. The average number of colonies per dish will then be determined. For each dose group, the average relative plating efficiency (relative survival) will be calculated, relative to the vehicle control group.

Labelling and coding of culture dishes: the dishes may be either labelled with a code in the beginning of the experiment or after staining and counting for the total number of colonies. The label should include the assay number, trial number, and dose level (pre-test) or dose code (main test).

2.4.5. Time schedule

1 d	feeder cell seeding
2 d	target cell seeding
3 d	test substance treatment initiation
10 d	end of treatment; fixation
10/11 d or later	staining and drying

2.4.6. Preparation of test cultures - feeder cells

Feeder cells are SHE cells that after X-ray radiation are no longer able to replicate but are still viable and of the same origin as the actual target cells. About 40 000 cells will be seeded per dish and used as a nutrient base for the relatively few target cells and to support the metabolic cooperation.

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

Cryopreserved SHE cells from a tested and approved lot will be thawed and grown to 50 - 90% confluency in growth flasks (2 - 4 days). On Day 1 of the assay, feeder cells will be detached and suspended in culture medium in a growth flask on wet ice. The cells will be x-ray irradiated to a point where they are still viable, yet no longer capable of replication (approximately 5 000 rad). Confirmation of this is made by preparing 5 plates containing only feeder cells. Following irradiation, the cells will be counted using a hemocytometer. The cell concentration is adjusted to 2 x 10^4 viable (determined by trypan blue exclusion method) cells/ml in culture medium and 2 ml of this suspension will be placed into each ø60 mm-culture dish. Dishes will be incubated at 37 ± 1°C in a humidified atmosphere of 10 ± 0.5% CO_2 for approximately 24 hours prior to addition of the target cells.

2.4.7. Preparation of test cultures - target cells

After seeding of the feeder cells, a second vial of SHE cells from the above lot is thawed and seeded in a growth flask and incubated at 37 ± 1°C in a humidified atmosphere of 10 ± 0.5% CO_2 for approximately five to 24 hours. After the five to 24 hour incubation period, the target cells will be detached, counted with a hemocytometer, and diluted with culture medium to a concentration that will yield approximately 25 - 45 colonies/dish. For the target cell adjusted dose groups, the required number of target cells/dish is determined from results of the cytotoxicity assay. The number of target cells seeded should achieve approximately 25 - 45 colonies/dish, with an optimum of 35 colonies/dish. Two ml of the target cell suspensions will be placed into each culture dish, containing 4 x 10^4 feeder cells. Dishes will be incubated at 37 ± 1°C in a humidified atmosphere of 10 ± 5% CO_2 for approximately 24 hours prior to test article treatment.

2.4.8. Treatment of the test cultures (7-day exposure)

Approximately 24 hours after the seeding of target cells, the test substance dosing solutions will be prepared (2x the final concentration) and 4 ml of the dosing solutions will be transferred to ø60 mm-dishes containing 4 ml of complete medium with feeder and target cells (final volume: 8 ml), and the cultures will be incubated for 7 days at 37 ± 1°C, 10 ± 0.5% CO_2 and ≥ 85% humidity.

2.4.9. pH and osmolality determination

The pH of the test article dosing solutions will be checked after at least four hours of undisturbed incubation at 37 ± 1°C with approximately 10 ± 0.5% CO_2 in the air. To achieve this, 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 to or greater than the highest concentration to be tested in the cytotoxicity assay. The pH will be determined using a standard pHmeter both at the time of preparation and after at least four hours of incubation.

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

2.4.10. Solubility

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

2.4.11. Fixing and staining of the colonies

- The complete medium will be poured out of the dishes. The dishes may be washed with approximately 5 ml of HBSS-CMF.

- The cells will be fixed with approximately 5 ml of methanol per dish for at least 10 minutes.

  After the removal of methanol the plates will be completely air-dried.

- The plates will be stained with approximately 3 - 5 ml of Giemsa solution (10% in purified water) for at least 10 minutes.

- After removal of the Giemsa solution, the dishes will be rinsed with tap water and air-dried.

3. EVALUATION

3.1. MORPHOLOGICAL CELL TRANSFORMATION

The stained dishes will be examined with a stereomicroscope, and individual colonies will be evaluated for MT.

Generally, for each test group ≥ 1 000 colonies will be evaluated for MT.

Sparse colonies are not scored for MT (but recorded in the spread sheet), 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 not recorded. Colonies at the edge of the plates can be scored for MT if clearly morphologically transformed.

A differentiation will be made between

- normal (non-transformed) colonies and
- transformed colonies.

3.1.1. Evaluation criteria

Normal (non-transformed) colonies contain cells in monolayer with an organized, often flowing growth pattern with minimal cell stacking. Since primary SHE cells represent diverse lineages, various normal colonies with different morphology exist in every dish:

- Epithelial-like normal colonies usually have more rounded cells with a smooth perimeter.
- Fibroblast-like normal colonies tend to have some random growth pattern (criss-crossing cells) on the perimeter of the colony.

Morphologically transformed colonies contain cells in an extensive, random-oriented, three-dimensional growth pattern with cell stacking and criss-crossing both at the colony center 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:

- Epithelial-like transformed colonies primarily exhibit extensive cell stacking.
- Fibroblast-like transformed colonies tend to have more basophilic cells with an extensive criss-crossing growth pattern.

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

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

3.1.2. Cytotoxicity

- **Relative Plating Efficiency (RPE)**

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

For the determination of the plating efficiency (PE), all colonies will be evaluated including those which are not scored for morphological cell transformation (*e.g.* sparse colonies on account of toxicity, incomplete colonies and colonies on the side of the dish).

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

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

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

- **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 are macroscopically observed and scored in three categories:

– normal;

– slightly reduced;

– greatly reduced

3.2. STATISTICS

For significance assessment Fisher's exact test or if applicable Cochrane-Armitage trend test will be applied.

3.3. ACCEPTANCE CRITERIA

- There should be a total of at least 1 000 colonies (for MT) per test group (= 40 dishes).
  - fewer than 1 000 colonies are acceptable if the dose group shows a significant increase in the transformation rate. However the average number of colonies per plate should not be less than 25.

- An average of 25 - 45 colonies per dish should be available for each test group.

- The plating efficiency of the negative / vehicle control should be $\geq$ 20%.
-
- In the feeder cells control dishes, no colonies formation should be observed. Feeder cells must be visible in the chemical treatment groups except if they are affected selectively by the compound.

- **0.6%** has been chosen as upper limit for the number of colonies with morphological cell transformations in the negative controls (untreated control / vehicle control). This value is based on published data and **it is consistent with historical data of each laboratory.**

- The positive control substance must lead to a statistically significant increase in the number of morphologically transformed colonies.

- There should be at least 5 scorable test article concentrations.

3.4. ASSESSMENT CRITERIA

- A test substance will be considered "negative/non-transforming" if the following criteria are met:
  - the percentage of morphologically transformed colonies in the test article treated groups is not statistically significant relative to the concurrent vehicle control or it is less than or equal to 0.6%.

- A finding will be assessed as "positive" if the following criteria are met:

  - a statistically significant increase in transformation frequency (above 0.6% MT) at least at two dose levels compared to the concurrent vehicle control or
  - a statistically significant increased frequency in morphologically transformed colonies (above 0.6% MT) only at a single dose level but with a general positive trend.

4. PREPARATION OF EMBRYONIC CELLS

SHE cells can be purchased from an experienced laboratory or prepared prior to the cell transformation assay. The cells will be stored in liquid nitrogen (5).

4.1. SHE CELLS

SHE cells are diploid and genetically stable primary cells derived from trunk tissue of 13 to 13.5 day-old embryos of Syrian golden hamsters. They consist of not yet completely differentiated, metabolically competent cells of different origin and type of tissue, have a finite lifespan in culture and show:

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

4.2. ISOLATION OF HAMSTER EMBRYOS

On day 13 of gestation, the females are sacrificed by CO_2. The ventral surface is disinfected with a 70% ethanol solution and the peritoneal cavity is opened using sterile instruments. Both uterine horns are removed and transferred to a petri dish (ø10 cm) containing approximately 30 ml of ice-cold wash solution. The embryos are removed and transferred to new petri dishes also containing approximately 30 ml of ice-cold wash solution. Subsequently, each embryo is decapitated, eviscerated and delimbed.

4.3. DISSOCIATION OF EMBRYONIC TISSUE

For the isolation of embryonic cells, the remaining tissue is transferred to another petri dish or a beaker on ice containing 20 - 30 ml ice-cold wash solution and minced into 3 - 5 mm pieces using scissors. To remove as many blood cells as possible, the minced tissue is transferred to a trypsinization flask containing about 100 ml of ice-cold wash solution and stirred with a magnetic stirrer at a slow stirring speed for about 5 minutes. After the tissue has settled, the supernatant is removed and replaced with approximately 50 ml of dissociation solution before gentle stirring for 5 minutes at room temperature. The tissue is then allowed to settle for 5 minutes before the supernatant is discarded.

For the collection of cells, 75 - 100 ml of dissociation solution is added to the cell tissue and stirred slowly for 10 minutes. After the tissue has settled (after 5 minutes), the liquid supernatant containing isolated SHE cells is transferred to 50 ml centrifuge tubes containing 2 ml of cold foetal bovine serum each. This dissociation step is repeated 3 - 4 times.

The 50 ml tubes with the cell suspension are centrifuged at 200x*g* for 10 minutes, the supernatant is discarded, and the cell pellet is resuspended in 5 ml of cell isolation medium in each case. The cells of all embryos are then pooled to obtain approximately 20 - 50 ml of cell suspension and stored on ice until seeded.

The number of viable cells is determined using trypan blue, and the cells are seeded in cell culture flasks (225 cm^2) with complete medium (containing antibiotics) at a density of about 1.3 x 10^5 per cm^2 (number of viable cells about 3 x 10^7 per flask). The cells are incubated at 37 ± 1°C, 10 ± 0.5% CO_2 and ≥ 85% humidity. The medium may be replaced with approximately 45 ml of fresh complete medium (without antibiotics) after 3 - 4 hours of incubation, and the cells are further incubated for 20 - 48 hours.

4.4. STORAGE / CRYOPRESERVATION OF SHE CELLS

When the cells are about 50 - 90% confluent, the culture medium is aspirated off from the flasks and the cells are rinsed twice with 10 ml of HBSS-CMF. Subsequently, 4 ml of detachment solution are added and the cells are incubated for approximately 5 minutes at 37 ± 1°C, 10 ± 0.5% CO_2 and ≥ 85% humidity. To stop the detachment process (trypsin activity), 1.0 ml of foetal bovine serum is added per flask, the cells are pipetted 3 - 4 times, pooled in sterile 50 ml centrifuge tubes and centrifuged at 200x*g* for 10 minutes at 2 - 8°C. The supernatant is discarded and the cell pellet is resuspended in complete medium (without antibiotics).

After the cell count has been determined (using trypan blue exclusion method), the cells are adjusted to a density of 5.0 x 10^6 cells/ml of complete medium for feeder cells and 2.0 x 10^6 cells/ml complete medium for target cells and diluted with 2X cryopreservation medium (15% DMSO) in a ratio of 1:1. The resulting cell suspensions (2.5 x 10^6 cells/ml and 1.0 x 10^6 cells/ml of complete medium containing 7.5% DMSO) are deep-frozen in 1.0 ml aliquots in cryovials at ≤ -70°C for 24 hours. Subsequently, the cultures are stored at approximately -196°C (liquid nitrogen vapor phase). The storage period should not exceed 24 months.

4.5. CHECKING OF THE SHE CELLS

Before being used, each new cell batch is checked for

- Plating efficiency (= colony forming ability): the number of cells to be seeded as target cells in order to achieve 25-45 colonies per plate,

- Spontaneous transformation rate: the number of transformed colonies in the solvent (negative) control should be within the historical negative control data,

- Morphological transformation with the standard carcinogen benzo[a]pyrene: the number of transformed colonies in the positive control should be significantly higher than that in the negative control.

Once the isolate passed the above criteria, they were distributed among the participating laboratories.

The cells were shipped in liquid nitrogen shipper.

5. REFERENCES

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Material

CULTURE REAGENTS	SOURCE	Cat. No. Lot. No.
DMEM-L Dulbecco´s, Modified Eagle´s Medium, LeBoeuf´s Modification	**Biochrom, Berlin (phase 1)** **Quality Biological (phase 2)**	**FZ 9995** **H0002** **112-125-101**
Foetal Bovine Serum	**HyClone Lab. Inc. Logan, UT (phase 1 and 2)**	**SH30071.03** **APB20666**
L-Glutamine (200 mM) [(a)]	**Biochrom, Berlin**	**K 0282** [(b)]
Pancreatin [(a)] (2.5%)	**Invitrogen, Corp. Carlsbad, CA**	**45720-018** [(b)]
Penicillin/Streptomycin [(a)] 10 000 U/ml, 10 000 µg/ml	**Biochrom, Berlin**	**A 2213** [(b)]
OTHER REAGENTS		
HBSS-CMF [(a)] Hank´s balanced salt solution calcium- magnesium free	**Biochrom, Berlin**	**L 2053** [(b)]
Trypsin [(a)] (0.25%)	**Biochrom, Berlin**	**L 2123** [(b)]
Trypsin/EDTA (1x) [(a)] (0.05% / 0.02%)	**Biochrom, Berlin**	**L 2143** [(b)]
Trypanblau-lsg 0.5% [(a)]	**Biochrom, Berlin**	**L 6323** [(b)]
DMSO [(a)]	**Merck Darmstadt**	**102952** [(b)]
FIXATIVE AND STAINING		
Methanol [(a)]	**Merck Darmstadt**	**106009** [(b)]
Giemsa [(a)]	**Merck Darmstadt**	**109204** [(b)]
CULTURE VESSEL		
Petri dishes 60 x 15 mm	**Corning Inc. NY**	**430166** [(b)]

[(a)] Other products of the same quality from different suppliers may be used.
[(b)] Every batch of these products can be used. **Also Falcon vessels can be used.**

12.3 Repeated experiments

BASF and Harlan CCR were requested to repeat two experiments due to the fact that they had conducted them using the wrong vehicle. This section reports the original experiments performed by these laboratories.

12.3.1 Harlan CCR: 2,4-diaminotoluene

In the first experiment by Harlan CCR (TA1), 2,4-Diaminotoluene was dissolved in DMEM-L. Table 31 shows the concentrations which were selected on the basis of the DRF test. The MTF values of the test chemical treated doses ranged from 0.08% to 0.44% and the VC value was 0.21%. None of the test chemical concentrations induced a statistically significant increase in MTF compared to the VC ($p \geq 0.05$).

Table 31: Transformation assay TA1 results from Harlan CCR, testing coded 2,4-diaminotoluene

Harlan CCR 2,4-diaminotoluene (µg/ml) TA1	Scorable colonies	RPE (%)	Colony density/size	Transformed colonies	MTF (%)	Fisher's exact test *p*-value (one-sided)
VC (DMEM-L)	1431	100	+	3	0.21	-
25	1417	96.7	+	3	0.21	0.6518
40	1316	89.9	+	1	0.08	0.9265
50	1261	78.4	++	4	0.32	0.4311
60	1373	59.8	+++	6	0.44	0.2336
70	1389	47.6	+++	6	0.43	0.2391
80	1143	36.3	+++	3	0.26	0.5465
PC	1428	97.9	+	32	2.24	<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.
** = $p < 0.01$ (one-sided Fisher's exact test)

The above results were presented at the "Phase II meeting on prevalidation of Cell Transformation Assay" (30-31 May 2007). Although a positive trend could be seen, the results were not statistically significant. Due to the differences in the vehicle choice (DMEM-L instead of DMSO) used compared to the other laboratories, the VMT requested that the assay should be repeated with DMSO as the vehicle. This repeated experiment (TA2) is reported in the Final Report for 2,4-diaminotoluene by Harlan CCR (18 October 2007) and in section 6.2.2.2.

12.3.2 BASF: 3-methylcholanthrene

In the first experiment by BASF (TA1), 3-methylcholanthrene was dissolved in DMEM-L. Table 32 shows the test concentrations which were evaluated based on solubility and cytotoxicity tests. The MTF values of the test chemical treated doses ranged from 0.10% to 0.57%. None of the test chemical concentrations had a significant increase in MTF compared to the VC ($p \geq 0.05$).

Table 32: Transformation assay TA1 results from BASF, testing coded 3-methylcholanthrene

BASF 3-methylcholanthrene (µg/ml) TA1	Scorable colonies	RPE (%)	Colony density/size	Transformed colonies	MTF (%)	Fisher's exact test *p*-value (one-sided)
VC (DMEM-L)	1174	100	+	3	0.26	-
20	1115	97.2	+	5	0.45	0.3352
40	1058	95.6	+	6	0.57	0.205
60[#]	984	90.4	+	1	0.10	0.9126
80	1314	77.4	+	4	0.30	0.5614
100	1489	70.5	+	7	0.47	0.2854
120	1619	73.1	+	9	0.56	0.1841
PC	1219	108.9	+	26	2.13	<0.0001**

[#] = less than 1000 scorable colonies
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)

These results were presented at the "Phase II meeting on prevalidation of Cell Transformation Assay" (30-31 May 2007). Due to the differences in the vehicle choice (DMEM-L instead of DMSO) and the dose range, above 10 µg/l compared to the other laboratories (the suggested dose range from the VMT was 0.01 - 10 µg/ml), the VMT requested that the assay should be repeated with DMSO as the vehicle and 10 µg/ml as the top dose. This repeated experiment (TA2) is reported in section 6.3.2.1 and is detailed in the amended report by BASF (26 September 2007).

12.4 Analysis of Phthalic anhydride by mass spectrometry

All laboratories produced a statistically significant increase in morphologically transformed colonies after treating the SHE cells with phthalic anhydride. However, based on the literature, phthalic anhydride is classified as a non-carcinogen. Therefore it was decided to verify that the chemical shipped to and used by the laboratories was indeed phthalic anhydride. According to Actions A1 and A2 of the minutes from the "Meeting on Prevalidation of Cell Transformation Assays (Balb/c 3T3 and SHE)" of 30-31 May 2007, coded phthalic anhydride samples were sent to ECVAM from J&JPRD and BioReliance for analysis.

The samples (Fluka product no. 80018, Lot no. 1173489) were analysed by mass spectroscopy and compared to a new lot of phthalic anhydride (Fluka product no. 80018, Lot no. 1339610) and to phthalic anhydride from another company (Sigma product no. 320064-25G, Lot no. 11908MC276).

The results of the analysis confirmed that all the coded samples returned to ECVAM were indeed phthalic anhydride (Figure 23). The peaks, corresponding to the masses of the various chemical complexes, were present in all samples. The analysis was carried out twice, firstly with water and then with DMSO. In both cases the patterns were very similar. No additional peaks were detected when the chemicals were dissolved in DMSO, demonstrating that the chemical is dissolved but not transformed in any way.

University of Metz also tested phthalic anhydride using the SHE pH 7.0 protocol, therefore the chemical used by University of Metz was also analysed by mass spectrometry, as can be seen in the MS traces (Figure 24). It showed exactly the same profile as phthalic anhydride form the other laboratories.

Figure 23: Mass spectrometry analysis of phthalic anhydride

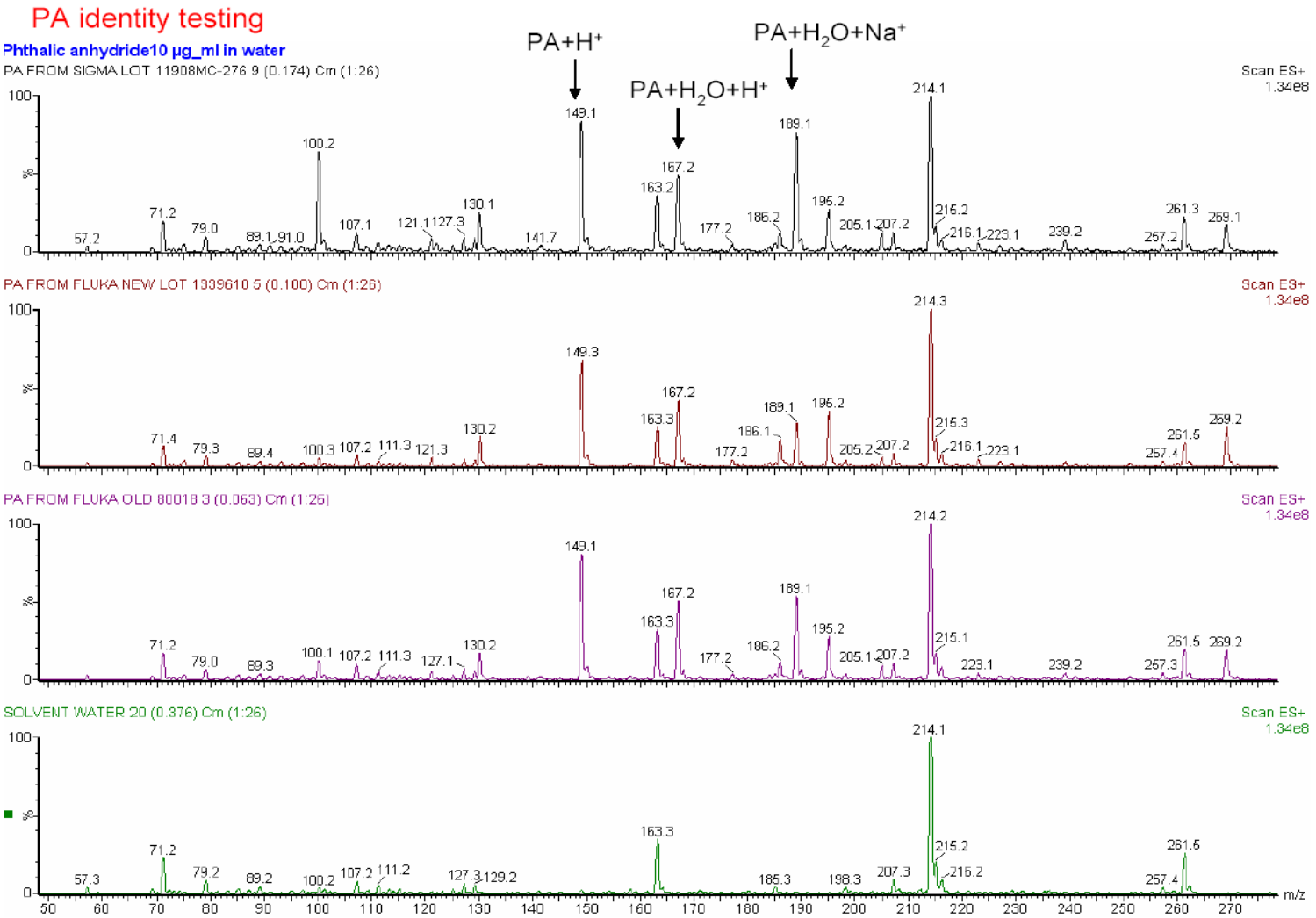

Figure 24: Mass spectrometry analysis of phthalic anhydride (samples from BioReliance and University of Metz)